PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, 15/82, 5/10, A01H 5/00 | A1

(11) International Publication Number: WO 96/38554

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/AU96/00335

(22) International Filing Date: 31 May 1996 (31.05.96)

(30) Priority Data:
PN 3285 31 May 1995 (31.05.95) AU

(71) Applicant *(for all designated States except US)*: QUEENSLAND UNIVERSITY OF TECHNOLOGY [AU/AU]; 2 George Street, Brisbane, QLD 4000 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DALE, James, Langham [AU/AU]; 10 Chalcot Road, Moggill, QLD 4070 (AU). HARDING, Robert, Maxwell [AU/AU]; 47 Akers Road, Lawnton, QLD 4501 (AU). DUGDALE, Benjamin [AU/AU]; 16 High Street, Milton, QLD 4064 (AU). BEETHAM, Peter, Ronald [AU/AU]; 29 Eighth Avenue, Kedron, QLD 4031 (AU). HAFNER, Gregory, John [AU/AU]; 80 Mountjoy Terrace, Wynnum, QLD 4178 (AU). BECKER, Douglas, Kenneth [AU/AU]; 34 David Street, Alderly, QLD 4051 (AU).

(74) Agent: KELLY, Robin, Thomas; Fisher Adams Kelly, Level 1, 349 Coronation Drive, P.O. Box 1477, Milton, QLD 4064 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: INTERGENIC REGIONS OF BANANA BUNCHY TOP VIRUS

(57) Abstract

A DNA molecule which is a partial fragment of an intergenic region of a BBTV component or alternatively which DNA molecule is derived from said intergenic region whereby the DNA molecule is capable of promoting, enhancing, regulating or modifying transcription of a non-BBTV gene.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

TITLE

"INTERGENIC REGIONS OF BANANA BUNCHY TOP VIRUS"

FIELD OF INVENTION

THIS INVENTION relates to DNA sequences of banana bunchy top virus (BBTV) and, in particular, to the intergenic regions of components 1 to 6.

BACKGROUND ART

Banana bunchy top disease (BBTD) is the most important virus disease of bananas (Dale, 1987, Advances in Virus Research **33** 301-325). The disease is widespread in Asia and the South Pacific and has limited distribution in Australia and Africa. It has not been reported from the Americas. The disease was originally assumed to be caused by a luteovirus as it was persistently aphid transmitted but not mechanically transmitted, induced yellows type symptoms and infected plants that had damaged phloem. However, recently 18-20 nm isometric virus-like particles (VLPs) have been purified from infected plants and have been demonstrated to be associated with the disease (Harding *et al.*, 1991, Journal of General Virology **72** 225-230; Thomas & Dietzgen, 1991, Journal of General Virology **72** 217-224; Wu and Su, 1990, Journal of Phytopathology **128** 153-160). Harding *et al.* (Harding *et al.*, 1991, Journal of General Virology **72** 225-230; Harding *et al.*, 1993, Journal of General Virology **74** 323-328) have isolated circular, single-stranded DNA of about 1 kb from these VLPs and cloned and sequenced one ssDNA component. This component, known as BBTV DNA component 1, had one large open reading frame (ORF) in the virion sense and encoded a putative replicase. This component was transmitted with the disease via aphids.

In Burns *et al.*, 1994, Arch Virol. **137** 371-380, they report the cloning and sequencing of a second component of BBTV. They found 0that a 93 nucleotide sequence was strongly conserved between the two ssDNA genomic components of BBTV. Two outwardly extending

degenerate primers were designed from this sequence and used in a polymerase chain reaction (PCR) with DNA extracted from purified BBTV virions. PCR amplified products consisting of at least seven distinct bands all approximately 1 kb and possibly representing full-length BBTV dsDNA were resolved. The PCR amplified products were cloned and the clones screened by restriction enzyme analysis. Four distinct restriction analysis groups were identified. This reference concluded that the genome of BBTV contains at least five components and that BBTV belongs to a previously undescribed group of plant viruses which may also contain subterranean clover stunt virus.

In Karan *et al.*, 1994, Journal of General Virology **75** 3541-3546, mention is made of BBTV component 1 from isolates from 10 different countries being cloned and sequenced and the sequences were subsequently aligned and compared. This analysis indicated two groups: the South Pacific group (isolates from Australia, Burundi, Egypt, Fiji India, Tonga and Western Samoa) and the Asian group (isolates from the Philippines, Taiwan and Vietnam). The mean sequence difference within each group was 1.9 to 3.0% and between isolates from the two groups were approximately 10%, but some parts of the sequences differed more than others. However, the protein encoded by the major open reading frame differed by approximately 5%. The region from the beginning of the stem-loop sequence to the potential TATA box was identical in all isolates except for a two nucleotide change in the Western Samoan isolate and a single change in that of the NSW isolate. These results, together with other evidence, suggest that BBTV has spread to bananas after the initial movement of bananas from the Asian Pacific regions to Africa and the Americas.

In Xie *et al.*, 1995, Phytopathology **85** 339-347, the Hawaiian isolate of BBTV was purified from infected banana cultivar Williams. Three single-stranded DNA (ssDNA) components were cloned and sequenced; they were named component 1, 3 and 4 respectively.

Component 1 is 1,110 nucleotides in length and shares 98% nucleotide sequence identity with the BBTV DNA component 1 of the Australian isolate as described in Harding *et al.* (1993) above. This component contains two open reading frames (ORF) capable of encoding a protein of 33.5 kDa, which may function as a replicase, and a protein about 15.2 kDa, with unknown functions. Component 3 is 1,057 nucleotides in length and does not contain any ORFs larger than 10 kDa. Component 4 is 1,017 nucleotides in length and potentially encodes a protein of 18.9 kDa. All three ssDNA components have a same stem-loop sequence and have a conserved non-coding region. The sequence of each of these three components is different from that of BBTV DNA components of two Taiwanese isolates. BBTV-specific clones were used in dot-blot hybridisation assays for detection of BBTV in plants using radioactive and non-radioactive probes. A polymerase chain reaction (PCR) assay was developed for detection of BBTV in banana samples and single aphids. Dot-blot hybridisation assays were as sensitive as enzyme-linked immunosorbent assay (ELISA) while PCR was 1,000 times more sensitive than dot-blot and ELISA assays for detection of BBTV in bananas.

Although so call intergenic regions of genomic components 1, 3 and 4 have been detected in Harding *et al.*, 1993 *supra*; and Xie and Hu, 1995, *supra*. The only charcteristic of such intergenic regions has been a stem-loop structure in component 1 as disclosed in Harding *et al.*, 1993, *supra*.

In an earlier application, (i.e. International Application PCT/AU95/00311) reference is made to BBTV components 3, 4 and 6 which are claimed *per se*. Regions outside the ORF regions (i.e. the so called "intergenic regions") have also been disclosed in this earlier application. Genomic components 1, 3 and 4 disclosed in Xie and Hu, 1995, *supra* correspond to components 1, 2 and 5 referred to in the earlier application PCT/AU95/00311.

SUMMARY OF THE INVENTION

Surprisingly, it has now been discovered that intergenic

regions of BBTV genomic components 1 - 6, or parts thereof, have sequences derived therefrom capable of promoting, enhancing, regulating or modifying transcription of non-BBTV genes.

The invention therefore includes within its scope, the aforementioned intergenic regions when used for promoting, enhancing, regulating or modifying transcription of a non-BBTV gene as well as parts thereof, sequences derived therefrom and mutants thereof *per se.*

In invention also includes within its scope a DNA molecule which is a partial fragment of an intergenic region of a BBTV component or alternatively which DNA molecule is derived from said intergenic region whereby the DNA molecule is capable of promoting, enhancing, regulating or modifying transcription of a non-BBTV gene.

The partial fragment is defined in this specification as being a sequence less than the full sequence of the intergenic region of a BBTV component but equal to or greater than a sequence of approximately 172 base pairs from a BBTV component. The sequence of said DNA molecule may be identical to the complementary sequence of the partial fragment of an intergenic region of a BBTV component.

The intergenic region is used in this specification to define the non-coding region of a BBTV component or the region outside the ORF in a BBTV component.

The sequence of the DNA molecule may be substantially identical or substantially complementary to the partial fragment of the intergenic region of BBTV components 1 - 6. Substantially is used in this specification to refer to sequences having variations up to 20%. The amount of sequence variation can be determined by standard hybridisation procedures or sequence comparison. The percentage of 20% is the variation shown with the region outside of the ORF of component 1 between different geographical isolates (Karan *et al.*, 1994, Journal of General Virology, **75**, 3541-3546; and U.S. Patent Application No. 08/202186). Both of these documents are herein incorporated by reference to support the claim of 20% variation of all components of

BBTV. The variation determined for component 1 of different geographical isolates is representative of the variation between each component from different geographical isolates. Thus variation up to 20% also applies to the sequences of components 2 through to 6 discussed below.

The term derived defines any sequence that has been changed, altered or modified by whatever procedure including mutagenesis from a fragment of the intergenic region of a BBTV component.

In particular, the sequence of the DNA molecule may be the BBTV intergenic region derived inserts of pBT6.1 (approximately 623 base pair fragment), pBT6.2 (approximately 351 base pair fragment), pBT6.3 (approximately 239 base pair fragment), and pBT6.4 (approximately 172 base pair fragment) from component 6; pBT1.1 (approximately 214 base pair fragment), pBT1.INT (approximately 980 base pair fragment) from component 1; pBT2.1 (approximately 855 base pair fragment) from component 2; pBT3.1 (approximately 526 base pair fragment) from component 3; pBT4.1 (approximately 659 base pair fragment) from component 4; and pBT5.1 (approximately 454 base pair fragment) from component 5. The DNA molecule may be 275 base pair region which includes the CR-M that is present in the insert of pBT6.1 but not in the insert of pBT6.2. The 275 base pair region may be a regulatory region. The DNA molecule may comprise the region between or including the CRSL region and the ATG of the open reading frame of any one of the BBTV components 1 to 6. The inserts of pBT1.1, pBT2.1, pBT3.1, pBT4.1, pBT5.1 and pBT6.1 are shown in FIG. 11. FIG. 12 shows the sequence of the insert of pBT1.INT. FIG. 13 shows the DNA sequence of the inserts of (a) pBT6.1; (b) pBT6.2; (c) pBT6.3; and (d) pBT6.4.

The non-BBTV gene may be any suitable gene such as GUS, NPTII, insecticide resistance gene, herbacide resistance gene or a growth promoting gene.

The DNA molecule may transcribe the gene in any suitable

prokaryote or eukaryote host. Preferably, the DNA molecule may transcribe the gene in a monocotyledon plant cell such as plant cells from *Musa spp (banana)*, wheat cells. The DNA molecule may transcribe the gene in a monocotyledon plant such as banana and wheat. Preferably the DNA molecule may transcribe the gene in a dicotyledon plant cell such as tobacco and zucchini cells. The DNA molecule may transcribe the gene in a dicotyledon plant such as tobacco and zucchini. The DNA molecule preferably transcribes the gene in cells of undifferentiated tissue in a dicotyledon plant.

In a second aspect, the invention is a DNA chimaeric vector or cassette having a DNA molecule as described above upstream of a gene of interest to enable the promoting, enhancing, regulating or modifying of transcription of the gene.

The chimaeric vector may be derived from pBI101.3. The vector may be any suitable construct mentioned below. The cassette may be any suitable construct mentioned below. The gene of interest may be any suitable gene as mentioned above. The chimaeric vector or cassette may be introduced into any suitable host including monocotyledon plant cells and dicotyledon plant cells for expression in the host.

The invention in a third aspect is a plant cell having a DNA molecule as described above.

The invention in a fourth aspect is a plant with the plant cells as described above.

The invention in a fifth aspect provides a method of expressing a non-BBTV gene in a plant cell using the DNA molecule as described above.

The invention will now be described with reference to preferred embodiments. However, these preferred embodiments are given by way of example. Example 1 describes the discovery and identification of the further three components of BBTV and is incorporated in the specification for convenience to allow a man skilled in the art to isolate these BBTV components. This information has essentially been

published in Burns *et al.*, 1995, Journal of General Virology, **76**, 1471-1482).

EXPERIMENTAL

EXAMPLE 1: BBTV COMPONENTS

METHODS

Synthesis and cloning of cDNA. Bananas with characteristic symptoms of banana bunchy top disease were collected from the Nambour region of S-E Queensland. BBTV particles were purified as described by Wu & Su, 1990, Journal of Phytopathology **128** 153-160 and Thomas & Dietzgen, 1991, Journal of General Virology **72** 217-224. Nucleic acid was extracted from virions as described by Francki & Randles, 1973, Virology **54** 359-368. Double stranded DNA was synthesised as described by Gubler & Hoffman, 1983, Gene **25** 263-269 using random hexamers (Bresatec) to prime first strand synthesis. The dsDNA was treated with mung bean nuclease (Promega) and ligated into *Sma*I digested plasmid vector pUC18 (Upcroft & Healey, 1987, Gene **51** 69-75). The plasmid was then used to transform *Escherichia coli* strain JM109 (Hanahan, 1983, Journal of Molecular Biology **166** 557-580) and potential recombinant clones were identified by screening on X-gal substrate (Vieira & Messing, 1982, Gene **19** 259-268)

Plasmids were isolated using the alkaline lysis method (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory). Inserts were excised by digestion with *Eco*RI/*Hind*III, electrophoresed in agarose gels and capillary blotted onto Hybond N+ (Amersham) using 0.4 M NaOH (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory). Inserts for use as DNA probes were purified from agarose gels using a Gene-Clean kit (Bresatec). DNA probes were labelled using a Ready-To-Go labelling kit (Pharmacia) as recommended by the manufacturer. Prehybridisations and hybridisations were done as

described by Burns *et al.*, 1994, Archives of Virology **137** 371-380.

Sequencing and sequence analysis. Mini preparations of respective BBTV clones were prepared by alkaline lysis followed by polyethylene glycol precipitation (Hattori & Sakaki, 1986, Analytical Biochemistry **152** 232-238). Sequencing was done using ^{35}SdATP and a Sequenase kit (US Biochemicals) as recommended by the manufacturer. Reaction products were electrophoresed in 8% (w/v) polyacrylamide gel containing 7 M urea. Gels were fixed, dried and exposed to X-ray film. Primers used for sequencing were either universal sequencing primers or 17-30 nt primers complementary to appropriate regions of the cloned viral DNA synthesised using an Applied Biosystems (ABI) PCR Mate and processed as recommended by the manufacturer.

PCR products for sequencing were purified from agarose gels using a Gene-Clean kit (Bresatec). DNA was sequenced using a Sequenase kit (USB) essentially as described by the manufacturer. Denaturation of template DNA (500 ng) was done by boiling following the addition of DMSO and 3 pmoles of sequencing primer.

Nucleotide sequences were analysed using the GCG analysis package version 8 available through the ANGIS computing facility at the University of Sydney, Australia. Nucleotide and amino acid sequences were aligned using the Clustal V software package (Higgins *et al.*, 1991, CABIOS **8** 189-191). Four DNA databases (GenBank, GenBank Weekly Updates, EMBL and EMBL weekly updates) and five protein databases (SwissProt, SwissProt Weekly Updates, PIR, GenPeptide Proteins and GenPeptides Weekly Updates) were searched for sequence homologies with BBTV nucleotide and deduced amino acid sequences using two database search analysis programs, FASTA (Pearson & Lipman, 1988, Proceedings of the National Academy of Sciences, USA **85** 2444-2448) and BLAST (Altschul *et al.*, 1990, Journal of Molecular Biology **215** 403-410).

PCR: Analysis and cloning. Using the respective

nucleotide sequences of clones (I) pBTRP-11, 20, 80 and 88 and (ii) pBTRP-P1 and P2 and the nucleotide sequences of BBTV components 1 (Harding *et al.*, 1993, Journal of General Virology **74** 323-328) and 2, three sets of immediately adjacent outwardly extending primers ((I) primer A: 5' GCATCCAACGGCCCATA 3'; primer B: 5' CTCCATCGGACGATGGA 3'; (ii) primer C: 5' TATTAGTAACAGCAACA 3'; primer D: 5' CTAACTTCCATGTCTCT 3'; (iii) primer E: 5' CGGGa/tATa/cTGATTGt/gGT 3'; and primer F: 5' TACa/tTTTGTCATAGc/tGT 3') were synthesised and used in a PCR with BBTV DNA as template as described by Burns *et al.*, 1994, Archives of Virology **137** 371-380). The amplified products were cloned using the TA cloning kit (Invitrogen) into the plasmid vectors pCRII or pCR2000 as recommended by the manufacturer or into T-tailed pUC19 and Bluescript (Marchuk *et al.*, 1990, Nucleic Acids Research **19** 1154). Recombinant clones were selected using X-gal substrate on Luria Bertani (LB) agar containing the appropriate antibiotic and plasmids isolated using the alkaline lysis method (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory). Clones with apparent full-length inserts (approximately 1 kb) were selected for sequencing.

Polarity of virion ssDNA. BBTV ssDNA was extracted, electrophoresed in agarose and capillary blotted onto duplicate nylon membranes (Harding *et al.*, 1993, Journal of General Virology **74** 323-328). For component 2, a DNA 3'-end labelling kit (Boehringer Mannheim) was used to prepare ^{32}P-labelled strand specific oligonucleotide hybridisation probes (primer BT2F5.30 (G): 5' GGTCCCCTTTAAGATTCCTTTCTTCGTCGC 3'; primer BT2R5.30 (H): 5' CGGAAAATGAATAAGTATGAGGTGAAAGAG 3'). Membranes were prehybridised and hybridised for 12 and 20 hours respectively in Rapid-hyb (Amersham) at 60^0 C. Filters were washed once with 1% SDS, 2xSSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at room temperature

followed by washes with 2xSSC at 65° C. Dried membranes were exposed to x-ray film at -80° C using intensifying screens.

For components 3, 4, 5 and 6, strand-specific RNA probes were used. Full-length RNA transcripts of full-length BBTV clones of each of the four components were synthesised using a riboprobe *in vitro* transcription kit (Promega) as recommended by the manufacturer.

RESULTS

Cloning and sequencing of five genomic components

Five new genomic components of BBTV were cloned and sequenced from two libraries, (I) a random primed library and (ii) a PCR library.

(I) Random Primed Library

A random primed library was generated from BBTV ssDNA extracted from purified virions. The resultant dsDNA was treated with mung bean nuclease, blunt-end ligated into *Sma*I cut pUC18 and cloned into *E. coli* JM109. This library was screened with ^{32}P-labelled DNA from BBTV virions, healthy bananas and the insert from pBT338 which was a partial clone of BBTV DNA component 1 (Harding *et al.*, 1991, Journal of General Virology **72** 225-230; Harding *et al.*, 1993, Journal of General Virology **74** 323-328).

BBTV DNA Component 2: Four clones, pBTRP-11, 20, 80 and 88, hybridised with BBTV virion DNA and with each other but not with healthy banana DNA or pBT338. The inserts from these clones were sequenced; pBTRP-20 and 88, each with inserts of 220 bp, had identical sequences; pBTRP-80 had an insert of 188 bp and had 148 bp sequence identical to pBTRP-20 and 88 and a further 40 bp sequence at one end that was unique; the sequence of the 115 bp insert of pBTRP-11 was identical to the equivalent region of pBTRP-20 and 88. Two immediately adjacent, outward extending primers, primers A and B, were designed from the overlapping sequence of the four clones such that these primers would prime the amplification of full length dsDNA copies of a circular

ssDNA molecule (Harding *et al.*, 1993, Journal of General Virology **74** 323-328). BBTV virion ssDNA was amplified by PCR using these primers and *Pfu* DNA polymerase and the product cloned into pCR2000. Four of the resultant clones were sequenced in both directions using universal forward and reverse primers and sequence specific primers. Three of these clones contained 1060 bp inserts and one clone contained a 1059 bp insert. The four clones had identical sequences except for nine single nucleotide changes including one deletion. Further, the sequences of the original four cDNA clones were found within the four PCR clones. The consensus sequence of this component, termed BBTV DNA component 2, was compiled (FIG. 1a) and compared with the sequence of BBTV DNA component 1 (Harding *et al.*, 1993, Journal of General Virology **74** 323-328); the two sequences were essentially different apart from two significant regions of homology.

BBTV DNA Component 6: A further two clones from the same random primed library, pBTRP-P1 and P2, also hybridised with labelled BBTV virion DNA but not with DNA from healthy bananas or pBT338. However, the inserts of these clones, both of approximately 1 kb, were digested with *Eco*RV whereas neither components 1 nor 2 had *Eco*RV sites. The two clones were partially sequenced using universal forward and reverse primers. The sequences of both clones were identical but clearly different to those of components 1 and 2. Again, two immediately adjacent, outwardly extending primers, primers C and D, were designed from the sequence and synthesised. BBTV virion ssDNA was used as a template with these two primers in a PCR reaction and the resultant product cloned into a T-tailed Bluescript vector. One apparent full length clone, pBT-P2A1, was selected and sequenced in both directions from subclones generated by exonuclease III digestion and universal forward and reverse, and sequence-specific primers. The final component 6 sequence of 1089 bp was then compiled (FIG. 1e)

(ii) <u>PCR Library</u>

When the sequences of components 1 and 2 were compared, two regions of homology were identified. The first region, later defined as the stem-loop common region (CR-SL) included the potential stem/loop sequence previously identified in component 1 (Harding *et al.*, 1993, Journal of General Virology **74** 323-328); the second region, which was contained within the region later defined as the major common region (CR-M), was a sequence of approximately 66 nucleotides 5' to the stem-loop sequence. It was hypothesised that all BBTV genomic components should contain a CR-M and therefore two immediately adjacent, outwardly extending degenerate primers, primers E and F, were designed from this region, synthesised and extended by PCR using BBTV virion ssDNA as a template (Burns *et al.*, 1994, Archives of Virology **137** 371-380). Seven products, each of approximately 1 kb, were resolved by polyacrylamide gel electrophoresis. The products were cloned into pCRII. The resultant clones were divided into three groups, groups B, C and D, on the basis that they hybridised with BBTV virion DNA but not DNA from healthy bananas and that each group had restriction patterns different to the other two groups and to components 1, 2 and 6 (Burns *et al.*, 1994, Archives of Virology **137** 371-380). One group, group A, had a restriction pattern indistinguishable from that of component 2 and it was later confirmed by sequencing that group A clones represented clones of component 2.

Each group of clones was assumed to represent a new and unique BBTV DNA component. For each group of clones, three clones (component 3) or four clones (components 4 and 5) were partially sequenced using universal forward and reverse primers. In each instance, all the clones within a group had identical sequences where these sequences overlapped except for one or two single nucleotide changes. Further, the sequences from each group were different to each other group and to the sequences of components 1, 2 and 6. One clone from each group or component was selected and fully sequenced in both directions. Importantly, each of these groups of clones were generated

using degenerate primers covering a sequence of 34 nucleotides derived from the conserved CR-M of components 1 and 2. The CR-M from components 1 and 2 was not fully conserved and thus it was expected that the hypothesised CR-M sequence would vary between other components. Therefore, converging primers unique to each component were designed and used to amplify a sequence including CR-M for each component from BBTV virion ssDNA. The resultant PCR product was sequenced directly using the two component specific converging primers.

BBTV DNA Component 3: Component 3 (Group C clone pBTP-64) was sequenced in both directions from the original clone and from subclones generated by exonuclease III digestion or restriction fragments using universal forward and reverse primers and three sequence-specific primers. Two additional converging primers were designed from this sequence to amplify a 380 bp product including the CR-M. The sequence of this product was identical to that of pBTP-64 except for five single nucleotide changes, four of which were in the sequence covered by the original degenerate primers and one outside this sequence at nucleotide 947. The final component 3 sequence of 1075 bp was then compiled (FIG. 1b).

BBTV DNA Component 4: Component 4 (Group D clone pBTP-62) was sequenced in both directions from the original clone and from subclones generated by exonuclease III digestion using universal forward and reverse primers and three sequence-specific primers. Two additional converging primers were designed from this sequence to amplify a 350 bp product including the CR-M. The sequence of this product was identical to that of pBTP-62 except for two single nucleotide changes in the sequence covered by the original degenerate primers. The final component 4 sequence of 1043 bp was then compiled (FIG. 1c).

BBTV DNA Component 5: Component 5 (Group B clone pBTP-129) was sequenced in both directions from the original clone and from subclones generated by exonuclease III digestion using universal forward and reverse primers and three sequence-specific primers. Two

additional converging primers were designed from this sequence to amplify a 290 bp product including the CR-M. The sequence of this product was identical to that of pBTP-129 except for four single nucleotide changes in the sequence covered by the original degenerate primers. The final component 5 sequence of 1018 bp was then compiled (FIG. 1d).

Orientation of genomic components and association with banana bunchy top disease

We have previously shown that the BBTV genome is encapsidated as single-stranded DNA (Harding *et al.*, 1991, Journal of General Virology **72** 225-230; Harding *et al.*, 1993, Journal of General Virology **74** 323-328). To determine the orientation of each component in virions, strand-specific DNA or RNA probes specific for each component were synthesised and hybridised with BBTV virion DNA. Component 2 specific probes were two 3' end-labelled 30mer oligonucleotides whereas probes specific for components 3, 4, 5 and 6 were SP6, T3 or T7 promoted ^{32}P-labelled RNA transcripts. For each component, the probes whose sequences were complementary to the component sequences presented in FIG. 1 hybridised strongly to BBTV virion DNA whereas the probes whose sequences were the same as the FIG. 1 sequences did not hybridise (FIG. 2). This indicated that each component was encapsidated as ssDNA and only in one orientation, that presented in FIG. 1.

Further, the strand- and component-specific probes that hybridised with BBTV virion ssDNA were used as probes to demonstrate that each component was associated with banana bunchy top disease. Plant DNA extracts from three (for component 2) or four (for components 3 to 6) different BBTV isolates and DNA from four healthy bananas was Southern blotted and hybridised with each probe. Each component-specific probe hybridised with a low molecular weight DNA of expected size in all the extracts from BBTV-infected bananas but did not hybridise with the extracts from healthy bananas (results not shown). This indicated that each component was clearly associated with the disease

and the virus.

Analysis of the BBTV genomic components

The sequences of the five BBTV genomic components presented here and the sequence of component 1 (Harding *et al.*, 1993, 2) were aligned and compared. Each of the six sequences were different except for two significant regions which had varying degrees of homology between all six components.

Stem-loop common region: We have previously identified a potential stem-loop structure in BBTV component 1 (Harding *et al.*, 1993, Journal of General Virology **74** 323-328) which had a loop sequence almost identical to the invariant loop sequence of geminiviruses (Lazarowitz, 1992, Critical Reviews in Plant Sciences **11** 327-349). An equivalent stem/loop structure was also found in components 2 to 6 (FIG. 3). Each component had an 11 nucleotide loop sequence of which 9 consecutive nucleotides were conserved between all components. Each component also had a 10 bp stem sequence of which 14 nucleotides were fully conserved. However, when all six components were compared, the region of homology extended up to 25 nucleotides 5' of the stem-loop structure and up to 13 nucleotides 3' of the stem/loop structure. The 5' 25 nucleotides were fully conserved between components 1, 3, 4 and 5. There were apparently two deletions in both components 2 and 6. In component 2, eight nucleotides were fully conserved with components 1, 3, 4 and 5 whereas in component 6, 16 nucleotides were conserved with these other components. The 13 nucleotides 3' of the stem-loop were fully conserved between all six components except for an apparent single nucleotide deletion in component 2. The sequence of up to 69 nucleotides including the stem-loop sequence was termed the stem-loop conserved region or CR-SL.

Major common region: The second common region was located at various distances 5' of the CR-SL and was called the Major Common Region or CR-M. This region varied in size from 65 nucleotides

in component 1 to 92 nucleotides in component 5 (FIG. 4). Component 1 apparently had the first 26 nucleotides of the CR-M deleted as well as a further single nucleotide deletion. Components 2, 3 and 4 had two single nucleotide deletions and component 6 had one single nucleotide deletion. Forty-five nucleotides were conserved between all components and 23 of the first 26 nucleotides, deleted in component 1, were conserved between components 2 to 6. Also in components 2 to 6, there was an almost complete 16 nucleotide direct repeat (ATACAAc/gACa/gCTATGA) from nucleotides 4 to 20 and 21 to 36. Further, a 15 nucleotide GC rich sequence (average of 86% GC) was located from nucleotides 78 to 92 and was 93% conserved between all components.

The sequence between the last nucleotide of the CR-M and the first nucleotide of the CR-SL varied in length from 22 nucleotides in component 1 to 233 nucleotides in component 2 (FIGS. 1 and 5). Interestingly, this sequence of 175 nucleotides in components 3 and 4 was 97% conserved between these two components.

Potential TATA boxes: A potential TATA box was identified in BBTV component 1 and was located 20 nucleotides 3' of the last nucleotide of the stem-loop and 43 nucleotides 5' of the start codon of the putative replicase gene (Harding *et al.*, 1993, Journal of General Virology **74** 323-328). Similar potential TATA boxes were also identified in components 2 to 6. In each of these components, the potential TATA box was a nine nucleotide sequence, CTATa/ta/tAt/aA, and was located downstream from the stem-loop sequence (FIG. 1). However, the sequence between the last 3' nucleotide of the stem-loop sequence and the potential TATA box was considerably longer in components 2 to 6 than in component 1 and varied from 157 nucleotides in component 5 to 227 nucleotides in component 4 (FIGS. 1 and 5).

Analysis of p tential polyadenylation signals

Six potential polyadenylation signals were identified associated with the 3' end of the major ORFs of components 3 to 6. A GT-rich region of 10 to 17 nucleotides was located between 0 and 23

nucleotides 3' of each of these polyadenylation signals and each GT-rich region contained the trinucleotide sequence TTG (FIG. 4). Only one potential polyadenylation signal identified in component 2 had a corresponding GT-rich region with the trinucleotide sequence TTG and this was located 233 nucleotides 3' of the nonanucleotide potential TATA box in the virion sense.

CONCLUSIONS

All six components share two common regions, the CR-SL and the CR-M, in the putative intergenic or untranslated region and five of the six components had one large ORF in the virion sense with associated potential TATA boxes and polyadenylation signals (FIG. 5). The CR-SL incorporated the conserved stem-loop structure. The loop sequence of 11 nucleotides was conserved in all BBTV components with the exception of two nucleotides and was similar to that present in nine geminiviruses (Lazarowitz, 1992, Geminiviruses: genome structure and gene function. 2), CFDV (Rohde *et al.*, 1990. Virology **176** 648-651) and a further BBTV component (Yeh *et al.*, 1994, Virology **198** 645 652. A model for implicating the loop sequence in rolling circle replication has been described for geminiviruses (Saunders *et al.*, 1993, DNA forms of the geminivirus - African cassava mosaic virus - consistent with the rolling circle mechanism of replication. IXth International Congress of Virology, Glasgow, August, 1993. Abstract P60-18). It is possible that the loop sequence in BBTV has a similar function. The stem-loop sequences were also highly conserved in all BBTV components and contained the pentanucleotide sequence TACCC which has been shown to be the site for initiation of viral strand DNA synthesis in wheat dwarf geminivirus (Heyraud *et al.*, 1993, EMBO Journal **12** 4445-4452).

The major common region (CR-M) was identified in all components and was located 3' of the major ORF (except for component 2 where no major ORF was identified) and 5' of the CR-SL (FIG. 5). Hexanucleotide repeats were identified within the CR-M in all components

except that of component 1. However, no function could be directly attributed to these repeats but they may be associated with, or part of promoter sequences. The CR-M also contained a 15 nt GC-rich sequence located at the 3' end and had the potential to form a small stem-loop structure. This GC-rich sequence also contained two direct GC-repeats which resembled the Sp1 binding sites found in promoters of genes in animal cells and viruses (Fenoll *et al.*, 1990, Plant Molecular Biology **15** 865-877). A similar promoter in the monocot-infecting maize streak geminivirus has been shown to be required for maximal rightward transcription and also appeared to bind maize nuclear factors in a non-cooperative manner (Fenoll *et al.*, 1990, Plant Molecular Biology **15** 865-877).

Karan *et al.*, 1994, Journal of General Virology **75** 3541-3546, reported that the component 1 CR-M sequence was highly conserved within the "South Pacific" group of BBTV isolates (96.5% homology) and within the "Asian" group of isolates (98.0% homology) but was highly variable between the two groups of isolates (68.0% homology). There was 76% between the CR-M sequences of the six different components of an Australian isolate reported here. Therefore, it will be important to determine the level of homology between the CR-M sequences of individual components from the different groups of isolates to see whether these sequences are highly conserved within groups of isolates but variable between groups and different components and further whether this has any biological significance.

The nucleotide length and sequence between the CR-M and CR-SL was dissimilar in four of the six components. However, in components 3 and 4, this 175 nucleotides region was 97% homologous and the 334 nucleotides from the 5' end of the CR-M to the 3' end of the CR-SL were 98% homologous. A similar large common region of 300 nucleotides has been found in geminiviruses and is identical between the A and B components of individual bipartite geminiviruses (Lazarowitz,

1992, Geminiviruses: genome structure and gene function. 2). In geminiviruses, this region included the stem-loop region. A region of homology was also found in five of the seven components of SCSV which included the stem-loop region (Surin *et al.*, 1993, The subterranean clover stunt virus genome consists of micro-chromosomes encoding single ORFs. IXth International Congress of Virology, Glasgow, August, 1993. Abstract P62-1) which is similar to the geminiviruses but different to four of the six BBTV components.

Components 3, 4, 5 and 6 all had one large ORF in the virion sense, 3' of the CR-SL. Each of these ORFs had potential conserved TATA boxes and polyadenylation signals associated with them (FIG. 8). The potential TATA boxes highly conserved with the nonanucleotide sequence CTATa/ta/tAa/tA which was essentially similar to that described by Bucher *et al.*, 1990, Journal of Molecular Biology **212** 563-578. The distance between the potential TATA box and the translation initiation codon varied in each component from 13 nucleotides in component 3 to 102 nucleotides in component 1. An ATGG translation initiation codon was identified in the five components encoding large ORFs. However, two possible translation initiation codons were identified in component 3, the first at nucleotide 213 (ATGT) and the second at nucleotide 227 (ATGG); the second initiation codon was in frame with the first. This would suggest that the second initiation codon is the correct codon; this could be verified by 5' RACE or N-terminal sequencing of the ORF translation product. GT-rich regions were identified 0 to 24 nucleotides 3' of each of the polyadenylation signals in components 1, 3, 4, 5 and 6. Each of these GT-rich regions contained the nucleotide sequence TTG. Both the polyadenylation signals in components 3 and 6 had these sequences. The combination of a consensus polyadenylation signal (Aa/tTAAa/t) and a 3' proximal GT-rich region containing the trinucleotide sequence TTG were only associated with the single major virion sense ORF in components 1, 3, 4, 5 and 6 and were not identified elsewhere in these sequences suggesting that each of these components

encoded a single gene. Similar sequences have been associated with many polyadenylation signals and may be required for efficient termination (Gil & Proudfoot, 1984, Nature **312** 473-474; Conway & Wickens, 1985, Proceedings of the National Academy of Sciences USA **82** 3949-3953).

EXAMPLE 2: MODIFICATION OF EXPRESSION WITH INTERGENIC REGIONS OF BBTV

MATERIALS AND METHOD

The Plasmids:

Agrobacterium-mediated Transformation

All potential promoter sequences derived from the BBTV genome were ligated into the *Agrobacterium* binary vector, pBI101.3 (Clontech). This plasmid has an antibiotic resistance cassette consisting of nopaline synthase promoter/NPTII gene (kanamycin resistance gene)/nopaline synthase (NOS) terminator and a "promoterless" β-glucuronidase gene (GUS) with a NOS terminator. The BBTV derived sequences were ligated separately into the unique *Bam*HI site 5' of the promoterless GUS gene. The plasmid, pBI121 (Clontech) was used as the control plasmid. This plasmid is identical to pBI101.3 except that it has the CaMV 35S promoter 5' of the GUS gene promoting GUS expression.

Micro-projectile bombardment

For transient analysis of BBTV promoter activity via micro-projectile bombardment, the BBTV promoter / GUS / nos cassette was sub-cloned into pGEM3zf$^+$ (Promega). As a positive control for most experiments the CaMV 35S promoter / GUS / nos cassette was similarly sub-cloned from pBI121 into pGEM3zf$^+$ (pGEM35S-GUS). Large quantities of these plasmids in a highly purified form were prepared for biolistics using Qiagen Plasmid Maxi Kit (Qiagen) as per manufacturers instructions.

Transformation

The plasmids, pBI121, pBI101.3 or pBI101.3 with BBTV derived sequences 5' of the GUS gene were introduced into a range of plant species and tissue types either by microprojectile bombardment, electroporation or *Agrobacterium*-mediated transformation (Horsch *et al.*, 1989, In: Plant Molecular Biology Manual, Gelvin *et al.* (eds.) Dordrecht

Kluwer Academic Publishers, pp A5/1-A5/9; Hauptmann *et al.*, 1987, Plant Cell Reports **6** 265-270; Last *et al.*, 1991, Theoretical and Applied Genetics **81** 581-588).

Assay for GUS expression is essentially the same as described in Jefferson, 1987, Plant Molecular Reporter 5(4): 387-405.

Preparation of BBTV Promoter Constructs

Primers:

BT6.1 (25mer)	5' gcctgcagagttgtgctgtaatgtt 3'
BT6.2 (24mer)	gcggatccgcttctgccttccgct
BT6.3 (25mer)	gccctgcagcatggacgtcagcaagg
BT3.1 (24mer)	gcctgcagactattgtatggaatg
BT3.2 (23mer)	gcggatccctatctagacactgg
BT4.1 (23mer)	gctctagaatgggtattgatgta
BT4.2 (25mer)	gcggatccttagctgcgtcctattt
BT5.2 (23mer)	gcggatccgacgagtgatttcgg
BT129V3.17 (17mer)	gttatcatggcatcgac
BT1R1.17 (17mer)	gaacaagtaatgacttt
BT1.F4.30 (30mer)	ggaagaagcctctcatctgcttcagagagc
BT1.INT.R28	ggatcctacatgacaatttaaatgaacc
BT1.INT.F25	aagcttataaaacgaaggcgatgaa

PCR Conditions

Sequential Steps:

1. 94°C × 5min
2. 94°C × 1min)
   42°C × 1min) × 40
   72°C × 1min)
3. 72°C × 10min

1. CLONING OF BBTV INTERGENIC REGIONS:

BBTV Component 6

Full length component six (1089bp)

1. Intergenic region was PCR amplified from BBTV6

clone P2A1 using primers BT6.1 (+ 746) and BT6.2 (+280).

2. The 623bp intergenic region was subcloned as a PstI and BamHI fragment into pUC19 (pUC6.1).

3. The intergenic region was cloned from pUC6.1 as a HindIII and BamHI fragment into pBI101.2 (pBT6.1).

4. The BBTV6 promoter / GUS / nos fragment from pBT6.1 was subcloned into pGEM3zf$^+$ as a HindIII and EcoRI fragment (pGEM6.1-GUS).

Generation of BBTV6 Intergenic Region 5' Deletions

Construction of pBT6.2

1. A 272bp 5' deletion of the BBTV6 intergenic region was generated by digestion of pUC6.1 with AccI, a restriction site present at +1018 in the component six circle, and PstI present in the 5' region of the multiple cloning site of pUC19.

2. The ends were filled using Klenow fragment and re-ligated to produce pUC6.2

3. The 351bp fragment was cloned from pUC6.2 as a HindIII and BamHI fragment into pBI101.3 (pBT6.2).

4. The BBTV6 promoter (351bp) / GUS / nos cassette was subcloned from pBT6.2 as a HindIII and EcoRI fragment into pGEM3zf$^+$ (pGEM6.2-GUS).

Construction of pBT6.3

1. A 384bp 5' deletion of the BBTV6 intergenic region was generated by PCR amplification from BBTV6 clone P2A1 using primers BT6.3 (+41) and BT6.2 (+280).

2. The 239bp intergenic region was cloned as a PstI and BamHI fragment into pUC19 (pUC6.3).

3. The intergenic region was cloned from pUC6.3 as a HindIII and BamHI fragment into pBI101.3 (pBT6.3)

4. The BBTV6 promoter (239bp) / GUS / nos cassette was subcloned from pBT6.3 as a HindIII and EcoRI fragment into

pGEM3zf+ (pGEM6.3-GUS).

Construction of pBT6.4

1. A 451bp 5' deletion of the BBT6 intergenic region was generated by digestion of pUC6.3 with BamHI and HaeIII (a restriction site present at position +108 in the BBTV 6 circle)

2. Ends were filled using Klenow fragment and the 172 bp fragment blunt-end cloned into the SmaI site of pBI101.3 (pBT6.4)

3. The BBTV6 promoter (172bp) / GUS / nos cassette was subcloned from pBT6.4 as a HindIII and EcoRI fragment into pGEM3zf+ (pGEM6.4-GUS).

BBTV Component 1

Full length component one (1111bp)

1. A 225bp fragment containing the BBTV1 intergenic region was PCR amplified from full-length BBTV1 clone using primers BT1R1.17 (+986) and BT1F4.30 (+129).

2. The resulting PCR product was digested with TaqI (a restriction site present at position +118 in the BBTV1 circle).

3. The resulting 214bp fragment was blunt ended using Klenow fragment and cloned into the SmaI site of pBI101.3 (pBT1.1).

4. The BBTV1 promoter (214bp) / GUS / nos cassette was subcloned from pBT1.1 as a HindIII and EcoRI fragment into pGEM3zf+ (pGEM1.1-GUS).

Note: A small ORF in BBTV-1 was identified by 3' RACE experiments. This ORF is located at position +430 to +555. Based on the location of this ORF another BBTV-1 intergenic promoter was isolated.

1. The large intergenic region based on the recently identified smaller ORF in BBTV-1 was PCR amplified from full length BBTV-1 clone using primers BT1.INT.R28 (+429) and BT1.INT.F25 (+560).

2. The resulting 980bp intergenic region was cloned upstream of the GUS reporter gene in pBI101.3 as a BamHI and HindIII fragment (pBT1.INT).

BBTV Component 2

Full length component two (1059bp)

BBTV2 full length double stranded replicative form, cloned into pUC19 as an XbaI fragment at position +361 (pUC-BT2) was obtained from Raktham Wanitchakorn

1. An 855bp intergenic fragment was generated from pUC-BT2 by digestion with XbaI and AccI (a restriction site present at position +565 in the component two circle). The AccI end was blunt ended using Klenow fragment and the XbaI site kept sticky.

2. This full intergenic region was directionally cloned into similarly prepared pGEM3zf$^+$ vector (pGEM2.1)

3. The intergenic region was cloned from pGEM2.1 as a HindIII and BamHI fragment into pBI101.3 (pBT2.1)

4. The BBTV2 promoter (866bp) / GUS / nos cassette was subcloned from pBT2.1 as a HindIII and EcoRI fragment into pGEM3zf$^+$ (pGEM2.1-GUS).

BBTV Component 3

Full length component three (1075bp)

1. Component 3 full intergenic region was PCR amplified from BBTV infected banana nucleic extract (Qld) using primers BT3.1 (+761) and BT3.2 (+212).

2. The 526bp intergenic region was subcloned as a PstI BamHI fragment into pGEM3zf$^+$ (pGEM3.1)

3. The intergenic region was cloned from pGEM3.1 as a HindIII and BamHI fragment into pBI101.3 (pBT3.1).

4. The BBTV3 promoter (526bp) / GUS / nos cassette was subcloned from pBT3.1 as a HindIII and EcoRI fragment into pGEM3zf$^+$ (pGEM3.1-GUS).

BBTV Component 4

Full length component four (1043bp)

1. Component 4 full intergenic region was PCR amplified from BBTV infected banana nucleic extract (Qld) using primers BT4.1 (+662) and BT4.2 (+278)

2. The 659bp intergenic region was subcloned as an XbaI and BamHI fragment into pGEM3zf$^+$ (pGEM4.1)

3. The intergenic region was cloned from pGEM4.1 as an XbaI and BamHI fragment into pBI101.3 (pBT4.1)

4. The BBTV4 promoter (659bp) / GUS / nos cassette was subcloned from pBT4.1 as an XbaI and EcoRI fragment into pGEM3zf$^+$ (pGEM4.1-GUS).

BBTV Component 5

Full length component five (1018bp)

1. Component 5 intergenic region was PCR amplified from BBTV diseased banana nucleic acid extract (Qld) using primers BT129V3.17 (+639) and BT5.2 (+230).

2. The resulting PCR product was digested with BamHI and AccI (a restriction site present at position +794 in the component 5 circle). The AccI site was blunt ended using Klenow fragment and the BamHI end kept sticky.

3. The resulting 454bp intergenic region was directionally sub-cloned into similarly prepared pGEM3zf$^+$ (pGEM5.1).

4. The intergenic region was cloned from pGEM5.1 as a HindIII and BamHI fragment into pBI101.3 (pBT5.1).

5. The BBTV5 promoter (454bp) / GUS / nos cassette was subcloned from pBT5.1 as a HindIII and EcoRI fragment into pGEM3zf$^+$ (pGEM5.1-GUS).

2. TRANSIENT ANALYSIS OF BBTV PROMOTER ACTIVITY VIA MICRO-PROJECTILE BOMBARDMENT OF Nic tiana tab cum (NT) CELL LINE

Preparation f NT C II Suspension

1. 25mL of NT cell suspension was subcultured into 75mL of fresh NT liquid media and shaken at 28°C under moderate light

2. Two days post subculture, 50mL of the actively growing NT culture was transferred to a 50mL Falcon tube and allowed to settle for 5-10min.

3. The resulting packed NT cell volume was resuspended in an equal volume of NT liquid media.

4. 200µL aliquots of the NT cell mix were spotted onto NT solid media and allowed to air dry for 3-4hrs.

5. NT spots were incubated at 28°C under moderate light for 2 days

6. NT spots were subjected to micro-projectile bombardment as described below. Five NT spots were shot per promoter construct.

7. Following biolistics NT spots were incubated at 28°C under moderate light for 3 days.

8. Promoter activity was qualitatively analysed by GUS staining one NT spot per promoter construct using X-glucuronide substrate. The remaining four NT spots were subjected to quantitative GUS fluorometric analysis using MUG substrate. These techniques were performed essentially as described Jefferson, 1987, Plant Molecular Reporter 5(4): 387-405.

Preparation of DNA-gold Suspension for Micro-projectile bombardment

1. Gold suspension was vortexed briefly and sonicated in ice water for 30sec.

2. DNA-gold suspension was prepared on ice by addition of 2µg of DNA, 25µL of $CaCl.2H_2O$ (2.5M) and 5µL of spermidine free base (0.1M) to 25µL of gold suspension (100mg/mL).

3. DNA-gold suspension was vortexed intermittently for 5min then left to settle on ice for 10min.

4. A 22µL volume of the supernatant was removed and

discarded. The remaining suspension was vortexed and a 5µL aliquot used for micro-projectile bombardment.

5. One preparation of gold was used per promoter construct. This DNA-gold suspension provided enough suspension to shoot five NT spots.

Shooting Conditions for the Particle Inflow Gun

25mm Hg vacuum

Helium pressure approximately 550Kpa

Platform height 10cm

Mesh used to protect target

Constructs Used

Tests: pGEM6.1-GUS, pGEM6.2-GUS, pGEM 6.3-GUS, pGEM6.4-GUS PGEM1.1-GUS, pGEM2.1-GUS, pGEM3.1-GUS, pGEM4.1-GUS, pGEM5.1-GUS

Controls: pGEM35S-GUS

Results: The results of the experiment are shown in FIG.

Comparison of BBTV6 Promoter Activity

1. The full intergenic region of BBTV component six has promoter activity comparable to that of the 800bp CaMV 35S promoter from pBI121

2. The 272bp 5' deletion (pGEM6.2-GUS) causes a significant increase in promoter activity which is maintained with a further 112bp 5' deletion (pGEM6.3-GUS).

3. Promoter activity is significantly reduced with a further 75bp 5' deletion (pGEM6.4-GUS).

Significance of Results:

The increase observed in promoter activity between plasmids pGEM6.1-GUS and pGEM6.2- GUS implies the 272bp region surrounding the CR-M may contain a putative down-regulatory sequence responsible for this reduction in promoter activity.

The levels of promoter activity observed between plasmids pGEM6.2-GUS, pGEM6.3-GUS and pGEM6.4-GUS indicates the majority

of the strong promoter activity associated with the BBTV6 intergenic region is associated with a 112bp region situated 3' of the CR-S/L. Importantly, this region contains a putative promoter motif TGA-1b (position +44), which contains the core sequence TGACGT, analogous to other promoter motifs and transcription factor binding domains.

Comparison of BBTV1-6 Promoters

1. BBTV-1 promoter has no activity in transient transformation of NT cell suspensions

2. BBTV-2 promoter has the highest promoter activity of the six BBTV components, with levels 2-3 fold greater than the 800bp CaMV 35S promoter derived from pBI121.

3. BBTV-3, -4, and -5 promoters are relatively weak with levels of activity about 50% less than the CaMV 35S promoter.

4. BBTV-6 full intergenic region has similar level of promoter activity as that of the CaMV 35S promoter.

Significance of Results:

The absence of promoter activity associated with the BBTV-1 intergenic region may indicate this promoter has a highly tissue specific expression pattern or requires transcription factors absent in tobacco nuclei. The identification of a putative promoter motif (Type-1 element of the wheat histone H3 gene) associated with S-phase specific cell expression within this promoter may imply its activity is restricted to actively dividing meristematic tissue types.

The high levels of promoter activity associated with the BBTV-2 intergenic region may make this sequence a potentially useful promoter to drive high level expression. Despite the fact that BBTV infects a monocot, it appears from this study BBTV2-6 promoters are active, to varying degrees, in a dicot system.

3. TRANSIENT ANALYSIS OF BBTV PROMOTER ACTIVITY IN OTHER PLANT SPECIES

Micro-projectile Bombardment of Cucumber

1. Cucumber pre-embryogenic callus was sub-cultured onto SQM2EV media 2 weeks prior to biolistic transformation.

2. Callus was transferred to small SQM2EV plates 4 days prior to biolistic transformation

3. Micro-projectile bombardment was done as previously above

4. GUS activity was observed in transformed callus via qualitative GUS staining using X-glucuronide substrate (2 days post biolistics)

Constructs Used: pBT6.1, pBT6.3, pBT1.1, pBI121

Results

1. Both pBT6.1 and pBT6.3 produced a similar number of blue foci (transformation events) as pBI121 following GUS staining.

2. pBT1.1 showed no evidence of promoter activity in cucumber callus (ie. no blue foci were observed)

Electroporation of Zucchini Protoplasts

Isolation of Zucchini Protoplasts

1. A 1.5 mL volume of embryogenic zucchini callus suspension was mixed with 20 mL of Enzyme Mix (E3) and incubated at 25°C in the dark on a slow shaker (30-50 RPM) for 5-6 hrs.

2. Protoplasts were isolated by passage through 450 µm, 105 µm and 51 µm sized sieves and centrifugation at 40-50 g for 5 min.

3. Proplasts were washed once in PWS solution and finally resuspended in a known volume of TBS.

4. Cell numbers were estimated using a calibrated slide counter and the volume adjusted to contain 1 x 10^6 protoplasts/mL.

5. 10 µg of plasmid DNA was added to 1 mL of protoplasts and incubated on ice for 10 min.

El ctroporation f Zucchini Protoplasts

1. The protoplasts: DNA mixture was electroporated in a BioRad electroporation cuvette (0.4 cm electrode gap) with 10 µg of plasmid DNA using a Gene Pulsar (BioRad) apparatus with 3 pulses of 300V, 10 msec pulse width and 100 msec pulse delay.

2. Protoplasts were incubated for a further 10 min on ice and pelleted by centrifugation at 1000 RPM in a microfuge.

3. Protoplasts were washed once in Culture Media 415A and finally resuspended in 1 mL of 415A.

4. Protoplasts were transferred to a 12 well microtitre plate and incubated for 48 hrs on a slow shaker in the dark.

5. Following incubation the protoplasts were harvested by centrifugation and assayed for GUS activity.

Fluorometric GUS Assay

1. A 5 µg quantity of extracted protoplast protein was used standardly for fluorometric assays (estimated using BioRad Protein Assay Reagent).

2. Volume was adjusted to 100 µL with protein extraction buffer and incubated with 100 µL of MUG substrate (2 mM) at 37°C for 30 min.

3. Reactions were stopped by addition of 1 mL of Na_2CO_3.

4. Enzymic activity was estimated against a 4-MU standard curve (0-500 nM) using a fluorometric spectrophotometer (excitation - 365 nm; emission - 455 nm; integration time - 10).

The GUS activity was analysed using a fluorometric assay described by Jefferson, 1987, Plant Molecular Reporter 5(4): 387-405.
Constructs Used: pBT6.3, pBT1.1, pBI121.

Results

CONSTRUCT	GUS ACTIVITY (pmol MU/min/mg protein)
pBI101.3	0
pBI121	10,000
pBT1.1	<1000
pBT6.3	22,000

No GUS activity was observed with the promoterless GUS binary vector (pBI101.3)

Levels of GUS activity driven by the 239bp fragment of BBTV6 intergenic region (pBT6.3) were 2-fold greater than the 800bp CaMV 35S promoter (pBI121)

Little to no GUS activity was observed from the BBTV1 promoter (pBT1.1)

Micro-projectile Bombardment of Wheat Cell Suspension

1. Wheat cell suspension was subcultured into 50mL WTL1 media 4 days prior to micro-projectile bombardment.

2. Wheat cells were pelleted by low speed centrifugation and resuspended in 10mL WTL1 media.

3. Cells were transferred to sterile filter paper immediately prior to bombardment

4. Micro-projectile bombardment was done as previously described.

5. Two days post-biolistics the transformed wheat suspension was subjected to GUS staining using X-glucuronide substrate

Constructs Used: pBT1.1, pBT2.1, pBT3.1, pBT4.1, pBT5.1, pBT6.1, pBI121, DM8052 (rice actin enhanced promoter driving GUS expression).

Results

1. Rice actin enhanced promoter produced >5000 blue

foci per transformation

2. pBI121 produced, on average approximately 70 blue foci per transformation

3. Of all the BBTV promoter constructs tested, only pBT2.1 was active, producing on average 100blue foci per transformation

Micro-projectile Bombardment of Banana Male Flower Embryogenic Callus

1. An embryogenic culture of Cavendish banana cv. "Williams" was generated using male flowers as described by Escalant *et al.*, 1994, In Vitro Cellular and Developmental Biology **30P**: 181-186. This culture was maintained in a temporary immersion system in liquid MP medium (Escalant *et al.*, 1994, In Vitro Cellular and Developmental Biology **30P**: 181-186).

2. Five days prior to micro-projectile bombardment the embryogenic callus was transferred to solid MP medium.

3. Tissue was subjected to micro-projectile bombardment as previously described.

4. Transient GUS activity was visualised by GUS staining using X-glucuronide substrate 2 days post biolistics.

Constructs Used: pGEM2.1-GUS, pGEM3.1-GUS, pGEM4.1-GUS, pGEM5.1-GUS, pGEM-Ubi (Maize ubiquitin promoter driving GUS expression).

Results

1. Maize ubiquitin promoter drives high level expression of GUS in this tissue type, with intensely blue staining foci or transformation events (the exact number of foci are difficult to determine due to intensity and number).

2. Of the BBTV promoter constructs tested, all promoters have shown low levels of activity (<10 blue foci per transformation). The approximate order of strength based on the number

of blue foci is: pGEM2.1-GUS (highest), pGEM4.1-GUS, pGEM3.1-GUS, and pGEM5.1-GUS (lowest).

Significance of Results

Results from transient analysis of BBTV promoter activity in alternative plant species indicate that the BBTV6 promoter (particularly the 239bp fragment, pBT6.3) appears to be active in undifferentiated cucurbit (dicot) tissue types.

Results from micro-projectile bombardment of wheat cell suspension imply only the BBTV2 promoter is active to a significant degree. This result indicates that at least one of the BBTV promoters is active to some extent in Graminaceae monocots.

Transient assays using banana embryogenic cultures indicates that the BBTV promoters tested to date have some level of activity in their host plant species. Again, the strongest of the BBTV promoters tested was that of BBTV component 2, which may reflect its importance as a potentially useful promoter in the future.

4. STABLE TRANSFORMATION OF NT CELL SUSPENSION BY AGROBACTERIUM INFECTION

1. Two to three days prior to transformation a 5mL LB culture containing 100µg/mL kanamycin was inoculated with the transformed Agrobacterium strain of interest, from 40% glycerol stock

2. NT cells were used 5-7 days post-subculture (4mL of this culture was used per transformation, with an additional 4mL for the control which received no bacteria). Note: duplicate transformation were performed for each construct.

3. 1µL of acetosyringone (20mM in ethanol) was added per mL of NT cells.

4. Using a 5mL pipette, the NT cells were pipetted in and out about 20 times to help induce lesions and enhance the transformation event.

5. 100µL of Agrobacterium culture (dense growth) was added to a microtitre plate containing 4mL of the treated NT cells, and

mixed thoroughly.

6. Plates were incubated for 3 days at 28°C under moderate light.

7. Post incubation, 10mL of NT liquid medium containing 500µg/mL carbenicillin (NTC) was added to each well.

8. Cells were centrifuged at 1K for 4min in 50mL Falcon tubes made up to volume with NTC.

9. Washes were repeated 2 times.

10. Cells were finally resuspended in 5mL NTC and 2mL plated onto 2 NTKC solid media (kanamycin 100µg/mL, carbenicillin 500µg/mL). Plates were incubated at 28°C under moderate light.

11. 3-4 weeks post infection transformants were subcultured individually and maintained under selection with fortnightly subculturing.

12. Promoter activity was observed by GUS staining using X-glucuronide substrate.

Constructs Used: pBT6.1 (3 NT lines) , pBT6.2 (3 NT lines) , pBT6.3 (2 NT lines) , pBT2.1 (25 NT lines)

Results

1. Strong GUS activity in stably transformed NT callus with all BBTV promoter constructs

2. Some variation in expression between different lines of BT2.1 transformed NT, most probably due to the position effect (position of DNA integration into host genome) and number of integrated copies.

Significance of Results

These experiments indicate that the BBTV6 and BBTV2 promoters are highly active in stably transformed tobacco undifferentiated cell types. These findings support results from micro-projectile bombardment, in which these promoters showed strong transient activity. Furthermore, these results confirm that activity from these promoters is not affected to a significant degree by stable integration in the host

genome.

5. STABLE TRANSFORMATION OF TOBACCO BY AGROBACTERIUM-MEDIATED INFECTION OF LEAF DISCS

1. Promoter binary constructs were introduced into Agrobacterium strain LBA4404 by electroporation

2. Transformed Agrobacterium were grown under kanamycin selection (100µg/mL) and confirmed to contain the promoter construct by a modified alkaline lysis procedure.

3. Two to three days prior to transformation a 5mL LB culture of the transformed Agrobacteria to be used was initiated from either a single colony or 40% glycerol stock, and shaken at 28°C.

4. The culture was diluted 1 in 20 with LB and transferred to a sterile 10mL vial.

5. Leaves from 3-6 week old tobacco (Nicotiana xanthii) were excised and cut into pieces 1cm × 1cm

6. These pieces were transferred to the diluted Agrobacterium culture and left to soak for 10-15 min.

7. Tobacco pieces were blotted dry on sterile filter paper, placed adaxial (upper) side down on MS104 medium and incubated at 28°C until a slight bacterial growth was visible (2-3 days).

8. Leaf pieces were transferred to MS104 selection media (100mL kµg/anamycin and 200µg/mL timentin), and incubated under moderate light at 28°C.

9. After approximately 2 weeks crown gall callus was visible at the site of infection . After a further 2-5 weeks shoots were apparent.

10. When well defined stems were visible, shoots were excised and placed in MS Rooting Media (100µg/mL kanamycin and 200µg/mL timentin).

11. Actively growing plantlets (with root systems) were maintained in culture under selection and putative transformants

subjected to qualitative GUS staining using X-glucuronide substrate.

12. In general 6-12 putative transformants were obtained per promoter construct.

Constructs used

Tests: pBT6.1, pBT6.2, pBT6.3, pBT6.4
pBT2.1, pBT2.2, pBT2.3, pBT2.4
pBT1.1, pBT3.1, pBT4.1, pBT5.1

Controls: pBI121

Results

1. Strong constitutive GUS expression in leaves, stems, and roots transformed with pBI121.

2. Weak phloem-limited GUS expression in leaf and root sections from about 10% of tobacco plantlets transformed with the BBTV promoter constructs. The remaining 90% of transformants displayed no GUS activity within leaves and roots.

Significance of Results

Results obtained from qualitative GUS staining of tissue from the majority (90%) of tobacco stably transformed with the BBTV promoter constructs, implies these promoters have little to no promoter activity in whole plants or differentiated tissues. The fact that some BBTV intergenic regions (components 2 and 6) show high levels of expression in transient systems (micro-projectile bombardment of *Nicotiana tabacum* cell line), indicates the activity of these promoters may be limited to undifferentiated cell types, or that these promoters are silenced in the transition from undifferentiated callus to differentiated cell types. This theory is further supported by experiments in which callus regenerated from leaves of stably transformed tobacco (showing no GUS activity) display varying levels of GUS expression upon staining.

The benefit of such a unique expression pattern may be in driving selective resistance, as most plant transformation systems rely on strong selection of transformed tissue during an undifferentiated cell

phase. Therefore a transformation cassette containing a BBTV promoter (component 2 or 6) driving NPTII expression would provide strong kanamycin resistance in transformed tissue during the early stages of plant transformation (callus phase), but once cells were differentiated (ie. formation of shoots, leaves, etc.) the promoter would be switched off. As the expression of antibiotic and herbicidal selection genes in transgenic plants is a major concern in certain countries, the use of such a promoter would be beneficial.

6. Analysis of BBTV Promoter Driving NPTII Expression

Generation of BBTV6-NPTII Construct

1. The 239bp fragment of BBTV component 6 intergenic region from pUC6.3 was cloned upstream of the 800bp NPTII gene with CaMV 35S terminator as a BamHI and PstI fragment.

2. The resulting BBTV6 promoter (239bp) / NPTII / CaMV 35S terminator cassette was sub-cloned as a SacI and XbaI fragment between the left and right borders of the Agrobacterium T-DNA in the binary vector pTAB5.

3. The CaMV 35S promoter (530bp) / GUS / CaMV 35S terminator (200bp) cassette from pGUS2 was cloned downstream as an EcoRI fragment (see FIG. 14).

4. The resulting vector pBT6.3-NPT detailed below was subsequently used for Agrobacterium-mediated transformation of tobacco (as previously described).

Analysis of Transformants

1. Eight putative transformants were obtained following Agrobacterium-mediated transformation of tobacco (*Nicotiana xanthii*) with the pBT6.3-NPT construct. These eight plants were selected in media containing kanamycin at a concentration of 100µg/mL.

2. Levels of NPTII expression from these plants were estimated using a quantitative NPTII ELISA Kit (5 Prime– 3 Prime) as per manufacturers instructions. Comparisons were made between tissue types (leaves and roots) and between promoters (CaMV 35S promoter

and nopaline synthase promoter)

R sults

The results of experiments are shown in FIG.

1. High levels of NPTII expression were obtained in both leaves and roots of plants containing the CaMV 35S promoter driving NPTII expression.

2. In general, NPTII levels in the eight plants expressing NPT under the control of the BBTV6 promoter were significantly lower than CaMV 35S promoter. Levels of NPTII expression in the leaves did not exceed 6ng NPTII/mg protein. Levels within the roots were slightly higher but did not exceed 12ng NPTII/mg protein. These levels of expression are comparable with the nos promoter.

Significance of Results

These experiments have shown that the 239bp fragment of the BBTV6 intergenic region is capable of driving selective resistance in stably transformed tobacco. As predicted, the levels of NPTII expression in the stably transformed plants (leaves and roots) were very low, but comparable with that of the nopaline synthase promoter.

7. REGULATORY ELEMENTS PRESENT IN THE BBTV INTERGENIC REGIONS

(I) TATA box

- present in all BBTV components and positioned upstream of the transcriptional start codon
- conserved nonanucleotide sequence CTATa/ta/tAa/tA
- responsible for the correct initiation of transcription

(ii) TGA1-a motif

- consensus sequence TGACGTAA
- as-1 binding motif involved in transcription regulation

(iii) TGA1-b motif

- hexamer binding motif with consensus sequence TGACGT

(iv) Promoter type 1 element of wheat histone H3 gene (hexamer motif)

- consensus sequence ACGTAA

- present immediately 3' of the CR-S/L in all BBTV components
- hexamer motif binds related TAF-1 and HBP-1 proteins
- linked to expression specifically in the S-phase of the cell cycle (Nakayama *et al.*, 1992, FEBS Letters, **300**: 167-170) suggesting promoter activity is limited to undifferentiated, actively dividing cells

(v) Adh1 US1

- consensus sequence CCACG
- binding factor unknown
- present in all BBTV intergenic regions (either in virion or complementary sense)

(vi) Adh1-US3

- consensus sequence CGTGG
- binding factor unknown
- present in all BBTV intergenic regions (either in virion or complementary sense)

8. PLANT TISSUE CULTURE SOLUTIONS AND MEDIA

MSO Medium	**NT Solid Media**
MS Salts myo-inositol 100µg/mL thiamine-HCl 10µg/mL nicotinic acid 1µg/mL pyridoxine-HCl 1µg/mL sucrose 3% TC agar 0.7% pH 5.7	NT liquid media TC Agar 0.7%
MS104 Medium	**NTC Media**
MSO benzyladenine 1µg/mL naphthaleneacetic acid 0.1µg/mL pH 5.7	NT liquid media carbenicillin 500µg/mL
MS Selection Media	**NTKC Media**
MS104 kanamycin 100µg/mL timentin 200µg/mL	NT solid media kanamycin 100µg/mL carbenicillin 500µg/mL

MS Rooting Media MSO with 0.6% TC agar kanamycin 100µg/mL timentin 200µg/mL	**SQM2EV Media** MS Salts Myo-inositol 1mg/mL sucrose 3% TC Agar 0.7% 2,4-D 10µM BAP 1.5µM
NT Liquid Media MS Salts Sucrose 3% MES 0.5µg/mL B1-Inositol 1µg/mL KH_2PO_4 180µg/mL 2,4-D 0.222µg/mL pH5.7	**WTL1 Media** MS Salts CAB A organics L-asparagine 5µg/mL L-glycine 10µg/mL Gibco coconut water 2% NZ amine 50µg/mL glucose 10mg/mL sucrose 20mg/mL mannitol 20mg/mL 2,4-D 2µg/mL kinetin 0.2µg/mL pH5.8

PWS SOLUTION - (1 Litre)

calcium chloride	735 mg
MES	639.6 mg
mannitol	109.3 g

pH 5.6

ENZYME MIX (E3)

2.0% cellulase (R-10)

0.5% macerozyme (R-10)

0.5% hemicellulase

1.0% pectolyase (Y23)

0.5% BSA

Made up to volume with PWS (pH5.6)

TBS SOLUTION - (200mL)

Trizma base	0.73g
sodium chloride	1.75g

calcium chloride	0.18g
mannitol	9.2g
pH 9.0	

9. SIGNIFICANCE OF THE CR-M

DNA sequence alignments of the BBTV components 1-6 CR-M are supplied. This region is thought to act as a putative primer binding site and suggested to play a role in BBTV replication. Although the CR-M of BBTV contains two direct GC-repeats which resemble the Sp1 binding sites found in the promoters of genes in animals and viruses (eg. maize streak virus) it appears this region has no promoter enhancing role. This hypothesis is supported by 5' deletion analysis of the BBTV6 intergenic region which has demonstrated that removal of a 272bp region including the CR-M produces a significant increase in promoter activity. Furthermore preliminary studies with the BBTV2 intergenic region have shown no decrease in promoter activity with removal of the CR-M.

10. CONCLUSIONS

We have demonstrated that the intergenic regions or DNA sequences of BBTV components 1 to 6 have promoter activity and that this activity varies from one intergenic region to another. Using transient and stable expression systems we have shown that the BBTV promoters appear to possess a tissue specific expression pattern which may be analogous to the site of BBTV replication and accumulation in its natural host. Furthermore we have identified potential regulatory elements present in at least one of the BBTV promoters (component 6) which appear to influence reporter gene expression.

BBTV is a virus that infects monocots of the genus *Musa*. It could be expected therefore that the promoters derived from BBTV would have the strongest activity in banana and potentially other monocots. Transient studies with wheat cell suspensions and banana embryogenic callus have shown some of the BBTV promoters are active to some extent in such systems. Importantly, we have also found that two of the BBTV promoters (BBTV2 and 6) drive high level transient expression in

dicotyledonous (tobacco, cucumber, and zucchini) undifferentiated tissue types.

LEGENDS

FIG. 1

The nucleotides sequences of BBTV DNA components 2, 3, 4, 5 and 6 and the deduced amino acid sequences of the major ORF of these components are given in FIGS. 1(a), (b), (c), (d) and (e) respectively. The potential TATA boxes are in bold and double underlined; the potential polyadenylation signals are in bold and underlined; the stem-loop structure is in italics and underlined, with the stem sequence arrowed; the CR-SL is underlined; the CR-M is in bold and italics; and the ORF is in bold.

FIG. 2

Determination of the virion-sense orientation of BBTV DNA components 2 to 6. Each blot was separately probed with either ^{32}P-labelled oligonucleotides (component 2) or full length RNA transcripts (components 3 to 6) specific for the virion- or complementary-sense strands of each respective component. Panel (a) blots were hybridised with probes complementary to the component sequences presented in FIG. 1 and Panel (b) blots were hybridised with probes of the same sequences presented in FIG. 1. Lane 1: full length clone of each respective component; Lane 2: healthy banana nucleic acid; Lane 3: DNA extracted from purified BBTV virions.

FIG. 3

The aligned stem-loop common regions (CR-SL) of BBTV DNA component 1 to 6. The stem-loop structure in each component is underlined and the loop sequence is in italics. Asterisks indicate nucleotides that are conserved between all components. Dots have been included in some sequences to maximise sequence alignment.

FIG. 4

The aligned major common regions (CR-M) of BBTV DNA components 1 to 6. The 15 nucleotide GC-rich sequence is underlined. Asterisks indicate nucleotides that are conserved between all components and

diamonds indicate nucleotides that are conserved between components 2 to 6 in the first 26 nucleotides covering the deletion in component 1. Dots have been included in some sequences to maximise sequence alignment and the imperfect repeat sequences are shown in italics.

FIG. 5

Diagrammatic representation of the proposed genome organisation of BBTV. (I) The general organisation of all components and (ii) a linear representation of each component.

FIG. 6

Various constructions of the intergenic region of component 1.

FIG. 7

Various constructions of the intergenic region of component 2.

FIG. 8

Various constructions of the intergenic region of components 3 and 4.

FIG. 9

Various constructions of the intergenic region of component 5.

FIG. 10

Various constructions of the intergenic region of component 6.

FIG. 11

Aligned nucleotide sequences of the intergenic regions of components 1 to 6 of BBTV. bbtvpro1 is the insert of pBT1.1; bbtvpro2 is the insert of pBT2.1; bbtvpro3 is the insert of pBT3.1; bbtvpro4 is the insert of pBT4.1; bbtvpro5 is the insert of pBT5.1; and bbtvpro6 is the insert of pBT6.1. Note in bbtvpro1 that Adh1 US1 is present at position 1004. With respect to bbtvpro2 note that TGA-1b is present at positions 952 and 1053, Adh1 US3 is present at position 137, G-BOX is present at position 225. With respect to bbtvpro3 note that Adh1 US1 is present at position 1069. With respect to bbtvpro4 note that TGA-1b is present at position 182; Adh1 US1 is present at position 1037; and G-BOX is present at positions 45, 63, 128 and 148. With respect to bbtvpro5 note that TGA-1b is present at position 96; Adh1 US1 is present at position 1012; and G-BOX is present

at positions 45 and 64. With respect to bbtvpro6 note that TGA-1a is present at position 1083; TGA-1b is present at position 44; Adh1 US1 is present at position 173; and G-BOX is present at position 46. Also note that the G-BOX is a plant promoter motif which is associated with transcription core sequence: CACGTG.

FIG. 12

The DNA sequence of the insert of pBT1.INT (982 base pair). Note that the sequence of the intergenic region of BBTV component 1 is based on the small open reading frame.

FIG. 13

The DNA sequence of the insert of (a) pBT6.1 (622 base pair); (b) pBT6.2 (351 base pair); (c) pBT6.3 (238 base pair); and (d) pBT6.4 (182 base pair). Note that the highlighted region in pBT6.3 is the sequence associated with the majority of promoter activity of the component 6 intergenic region. This region is removed in pBT6.4.

FIG. 14

BBTV6-NPTII construct.

CLAIMS:

1. A DNA molecule which is a partial fragment of an intergenic region of a BBTV component or alternatively which DNA molecule is derived from said intergenic region whereby the DNA molecule is capable of promoting, enhancing, regulating or modifying transcription of a non-BBTV gene.

2. A DNA molecule as claimed in claim 1 wherein the DNA molecule has a sequence that is substantially identical or substantially complementary to a sequence of the intergenic region of a BBTV component.

3. A DNA molecule as claimed in claim 1 wherein the DNA molecule has a sequence which has up to 20% variation from a substantially identical or a substantially complementary sequence of the intergenic region of a BBTV component.

4. A DNA molecule as claimed in claim 1 wherein the DNA molecule has a sequence that is substantially identical to any one of the sequences shown in FIGS. 1, 3, 4, 11, 12 or 13.

5. A DNA chimaeric vector or cassette having a DNA molecule as claimed in any one of the preceding claims and being upstream of a gene of interest to enable the promoting, enhancing, regulating or modifying of transcription of the gene of interest.

6. A DNA chimaeric vector or cassette as claimed in claim 5 wherein the DNA chimaeric vector or cassette includes a BBTV6-NPTII gene construct shown in FIG. 14.

7. A method of expressing a non-BBTV gene in a plant cell using the DNA molecule as claimed in any one of the claims 1 to 4.

8. A method of expressing a non-BBTV gene as claimed in claim 7 wherein the plant cell is part of a monocotyledon or dicotyledon plant.

9. A plant cell having the DNA molecule as claimed in claims 1 to 4.

10. A plant cell as claimed in claim 9 wherein the plant cell is part of a monocotyledon or dicotyledon plant.

11. A plant having a plant cell as claimed in claim 9 or 10.

12. A plant as claimed in claim 11 which is a monocotyledon plant.

13. A plant as claimed in claim 11 which is a dicotyledon plant.

14. A DNA molecule as claimed in any one of claims 1 to 4 wherein the DNA molecule can express a non-BBTV gene in a monocotyledon plant cell.

15. A DNA molecule as claimed in any one of claims 1 to 4 wherein the DNA molecule can express a non-BBTV gene in a monocotyledon plant.

16. A DNA molecule as claimed in any one of claims 1 to 4 wherein the DNA molecule can express a non-BBTV gene in a dicotyledon plant cell.

17. A DNA molecule as claimed in any one of claims 1 to 4 wherein the DNA molecule can express a non-BBTV gene in a plant cell from undifferentiated tissue of a dicotyledon plant.

(a)

```
1    GGCGCTGGGG CTTATTATTA CCCCCAGCGC CGGGACGGGA CATGGGCTTT TTAAATGGGC
61   TTTGCGAGTT TGAACAGTTC AGTATCTTCG TTATTGGGCC AACCCGGCCC AATAATTAAG
121  AGAACGTGTT CAAATTCGTG GTATGACCGA AGGTCAAGGT AACCGGTCAA CATTATTCTG
181  GCTTGCGCAG CAAGATACAC GAATTAATTT ATTAATTCGT AGGACACGTG GACGGACCGA
241  AATACTCTTG CATCTCTATA AATACCCTAA TCCTGTCAAG GATAATTGCT CTCTCTCTTC
301  TGTCAAGGTG GTTGTGCTGA GGCGGAAGAT CGCCAGCGGC GATCGTCGGA ACGACCTGCA
361  TCTAGAGAGG CGGCGAGGAA ACTACGAAGC GTATATCGGG TATTTATAGA CTTATAGCGT
421  AGCTAGAAGT ATACACTGTA CAGATATTGT ATCTTGTAAA TTACGAAGCA ATTCGTATTT
481  GATATTAATA AAACAACTGG GTTTGTTAAT GTTTACATTA ACTAGTATCT TATATGTACA
541  AATTAAAATA CAGTATACGG AACGTATACT AACGTAAAAA TTAAATGATA GGCGAAGCAT
601  GATTAACAGG TGTTTAGGTA TAATTAACAT AATTATGAGA AGTAATAATA ATACGGAAAA
661  TGAATAAGTA TGAGGTGAAA GAGGAGATAT TAGAATATTT AAAAACCCAA TTATATTATT
721  TTGGAACGAA ATACAACACG CTATGAAATA CAAGACGCTA TGACAAATGT ACGGGAATAT
781  GATTGTGTAT CTTAACGTAT AAGGGCCGCA GGCCCGTCAA GTTGAATGAA CGGTCCAGAT
841  TAATTCCTTA GCGACGAAGA AAGGAATCTT AAAGGGGACC ACATTAAAGA CAGCTGTCAT
901  TGATTAAATA AATAATATAA TAACCAAAAG ACCTTTGTAC CCTTCCTAAT GATGACGTAT
961  AGGGGTGTCC CGATGTAATT TAACATAGCT CTGAAAAGAG ATATGGGCCG TTGGATGCCT
1021 CCATCGGACG ATGGAGGTTG AATGAACTTC TGCTGACGTA
```

FIG. 1

(b)

```
1    AGCGCTGGGG ACTATTATTA CCCCCAGCGC TCGGGACGGG ACATGGGCTA ATGGATTGTG

61   GATATAGGGC CCAAAGGGCC CGTTTAGATG GGTTTTGGGC TCATGGGCTT TATCCAGAAG

121  ACCAAAAACA GGCGGGAACC GTCCCAAATT CAAACTTCGA TTGCTTGCCC TGCAACGCAT

181  CTAGAAGTCT ATAAATACCA GTGTCTAGAT AGATGTTCAG ACAAGAAATG GCTAGGTATC
                                       M  F  R  Q  E  M  A  R  Y  P

241  CGAAGAAATC CATCAAGAAG AGGCGGGTTG GGCGCCGGAA GTATGGCAGC AAGGCGGCAA
       K  K  S  I  K  K  R  R  V  G  R  R  K  Y  G  S  K  A  A  T

301  CGAGCCACGA CTACTCGTCG TCAGGGTCAA TATTGGTTCC TGAAAACACC GTCAAGGTAT
       S  H  D  Y  S  S  S  G  S  I  L  V  P  E  N  T  V  K  V  F

361  TTCGGATTGA GCCTACTGAT AAAACATTAC CCAGATATTT TATCTGGAAA ATGTTTATGC
       R  I  E  P  T  D  K  T  L  P  R  Y  F  I  W  K  M  F  M  L

421  TTCTTGTGTG CAAGGTGAAG CCCGGAAGAA TACTTCATTG GGCTATGATC AAGAGTTCTT
       L  V  C  K  V  K  P  G  R  I  L  H  W  A  M  I  K  S  S  W

481  GGGAAATCAA CCAGCCGACA ACCTGTCTGG AAGCCCCAGG TTTATTTATT AAACCTGAAC
       E  I  N  Q  P  T  T  C  L  E  A  P  G  L  F  I  K  P  E  H

541  ACAGCCATCT GGTTAAACTG GTATGTAGTG GGGAACTTGA AGCAGGAGTC GCAACAGGAA
       S  H  L  V  K  L  V  C  S  G  E  L  E  A  G  V  A  T  G  T

601  CATCAGATGT TGAATGTCTT TTGAGGAAGA CAACCGTGTT GAGGAAGAAT GTAACAGAGG
       S  D  V  E  C  L  L  R  K  T  T  V  L  R  K  N  V  T  E  V

661  TGGATTATTT ATATTTGGCA TTCTATTGTA GTTCTGGAGT AAGTATAAAC TACCAGAACA
       D  Y  L  Y  L  A  F  Y  C  S  S  G  V  S  I  N  Y  Q  N  R

721  GAATTACATA TCATGTTTGA TATGTTTATG TAAACATAAA CTATTGTATG GAATGAAATC
       I  T  Y  H  V  *

781  CAAATAACAT ACAACACGCT ATGAAATACA AGACGCTATG ACAAAAGTAC TGGTATATGA

841  TTAGGTATCC TAACGATCTA GGGCCGAAGG CCCGTGAGCA ATATGCGTCG AAATAATGTT

901  TAACAAACAA ATATACATGA TACGGATAGT TGAATACATA AACAACGAGG TATACAATAC

961  AACAAACTGT TGTAAAGAAA TAAAAAATAA GAAGAGAGAG TATATTTGTG TCGGATAAGC

1021 ATCACACCCA CCACTTTAGT GGTGGGCCAG ATGTCCCGAG TTAGTGCGCC ACGTA
```

(c)

```
                  ⇒                    ⇐
1    AGCGCTGGGG CTTATTATTA CCCCCAGCGC TCGGGACGGG ACATCACGTG CGTCAACAAA

61   TGCACGTGAC TGATATAAGG GACATAACGG GTTTAGATAA CGGTTTATGC GGATTAGAAT

121  ATAACGTCAC GTGTGAAAGC CGAAAGGCAC GTGACGAAGA CAAATGGATT GAATAAACAT

181  TTGACGTCCG GTAGCTTCCG AAGGAAGTAA GCTTCGCGGC GAAGCAAACC ATTTATATAT

                                                 ⩛
241  TTGCGTAGGC TTGCGGCCTA TAAATAGGAC GCAGCTAAAT GGCATTAACA ACAGAGCGGG
                                          M  A  L  T  T  E  R  V

301  TGAAACTATT CTTTGAATGG TTTCTGTTCT TTGGAGCAAT ATTTATTGCG ATTACAATAT
       K  L  F  F  E  W  F  L  F  F  G  A  I  F  I  A  I  T  I  L

361  TATATATATT GTTGGTTTTG CTCTTTGAGG TACCCAGGTA TATTAAGGAG CTCGTGAGGT
       Y  I  L  L  V  L  L  F  E  V  P  R  Y  I  K  E  L  V  R  C

421  GTTTGGTAGA ATACCTGACC AGACGACGTG TATGGATGCA GAGGACGCAG TTGACGGAGG
       L  V  E  Y  L  T  R  R  R  V  W  M  Q  R  T  Q  L  T  E  A

481  CAACTGGAGA TGTAGAGATC GGCAGAGGTA TTGTGGAAGA CAGACGAGAT CAAGAACCGG
       T  G  D  V  E  I  G  R  G  I  V  E  D  R  R  D  Q  E  P  A

541  CTGTCATACC ACATGTATCT CAGGTAATCC CTTCTCAACC AAATAGAAGG GATGATCAAG
       V  I  P  H  V  S  Q  V  I  P  S  Q  P  N  R  R  D  D  Q  G

601  GAAGACGAGG AAACGCTGGA CCTATGTTCT AATACACGGT ATATTAATAT ACGAAATATA
       R  R  G  N  A  G  P  M  F  *

661  AATGGGTATT GATGTAAATG ATCATACATA ATATATGTAT GATAATGAAA CATATTGTAA

721  TATGTGAATT GTAAACGAGA GTTGTATGTA TAAAACATAC AACACGCTAT GAAATACAAG

781  ACGCTATGAC AAAAGTACTG GTATATGATT AGGTATCCTA ACGATCTAGG GCCGAAGGCC

841  CGTGAGCAAT ATGCGTCGAA ATAATGTTTA ACAAACAAAT ATACATGATA CGGATAGTTG

901  AATACATAAA CAACGAGGTA TACAATACAA CAAACTGTTG TAAAGAAATA AAAAATAAGA

961  AGAGATAGTA TATTTGTGTT GGATAAGCCT TGCAACCACC ACTTTAGTGG TGGGCCAGAT

1021 GTCCCGAGTT AGTGCGCCAC GTA
```

(d)

```
                ⇒                                ⇐
1   AGCGCTGGGG CTTATTATTA CCCCCAGCGC TCGGGACGGG ACATCACGTG CAACTAACAG

61  ACGCACGTGA GAATGCAGTA GCTTGCAGCG AAAGATAGAC GTCAACATCA ATAAAGAAGA

121 AGGAATATTC TTTGCTTCGG CACGAAGCAA AGGGTATAGA TATTTGTTCG AGATGCGAAA

                                                                    v
181 ATGGAGGCTA TTTAAACCTG ATGGTTTTGT GATTTCCGAA ATCACTCGTC GGAAGAGAAA
                                                                  -  M

241 TGGAGTTCTG GGAATCGTCT GCCATGCCTG ACGATGTCAA GAGAGAGATT AAGGAAATAT
      E  F  W   E  S  S   A  M  P  D   D  V  K   R  E  I   K  E  I  Y

301 ATTGGGAAGA TCGGAAGAAA CTTCTGTTCT GTCAGAAGTT GAAGAGCTAT GTCAGAAGGA
      W  E  D   R  K  K   L  L  F  C   Q  K  L   K  S  Y   V  R  R  I

361 TTCTTGTTTA TGGAGATCAA GAGGATGCCC TTGCCGGAGT GAAGGATATG AAGACTTCTA
      L  V  Y   G  D  Q   E  D  A  L   A  G  V   K  D  M   K  T  S  I

421 TTATTCGCTA TAGCGAATAC TTGAAGAAAC CATGTGTGGT AATTTGTTGT GTTAGCAATA
      I  R  Y   S  E  Y   L  K  K  P   C  V  V   I  C  C   V  S  N  K

481 AATCAATTGT GTATAGGTTA AACAGCATGG TGTTCTTTTA TCATGAATAC CTTGAAGAAC
      S  I  V   Y  R  L   N  S  M  V   F  F  Y   H  E  Y   L  E  E  L

541 TAGGTGGTGA TTACTCAGTA TATCAAGATC TCTATTGTGA TGAGGTACTC TCTTCTTCAT
      G  G  D   Y  S  V   Y  Q  D  L   Y  C  D   E  V  L   S  S  S  S

601 CGACAGAGGA AGAAGATGTA GGAGTAATAT ATAGGAATGT TATCATGGCA TCGACACAAG
      T  E  E   E  D  V   G  V  I  Y   R  N  V   I  M  A   S  T  Q  E

661 AGAAGTTCTC TTGGAGTGAT TGTCAGCAGA TAGTTATATC AGACTATGAT GTAACATTAC
      K  F  S   W  S  D   C  Q  Q  I   V  I  S   D  Y  D   V  T  L  L

721 TCTAATGTAA TATCCATTAT CATCAATAAA ATAATGGAAT GTTGATTATG TATTTATCAT
    *

781 AAATACATAA TGGTATACGT ATAGCATAAA ATACATTAAC CAACATACAA CACACTATAA

841 AATACAACAC GCTATGACAA ATGTACGGGT ATATGATTGG GTTATATTAA CCCCTTAAGG

901 GCCGAAGGCC CGTTTAAATA TGTGTTGGAC GAAGTCCAAA CACAAAAAAG TAAGCAGAAC

961 AACGGAATAA TATGAGCTGG CAACGTAGGG TCCATGTCCC GAGTTAGTGC GCCACGTA
```

(e)

```
                ⇒                                   ⇐
1    AGCACGGGGG ACTATTATTA CCCCCCGTGC TCGGGACGGG ACATGACGTC AGCAAGGATT

61   ATAATGGGCT TTTTATTAGC CCATTTATTG AATTGGGCCG GGTTTTGTCA TTTTACAAAA

121  GCCCGGTCCA GGATAAGTAT AATGTCACGT GCCGAATTAA AAGGTTGCTT CGCCACGAAG

181  AAACCTAATT TGAGGTTGCG TATTCAATAC GCTACCGAAT ATCTATTAAT ATGTGAGTCT
                                              v
241  CTGCCGAAAA AATCAGAGC GAAAGCGGAA GGCAGAAGCG ATGGATTGGG CGGAATCACA
                                               M  D  W  A  E  S  Q

301  ATTCAAGACC TGTACTCATG GATGCGATTG GAAGAAGATA TCATCGGATT CAGCCGATAA
      F  K  T  C  T  H  G  C  D  W  K  K  I  S  S  D  S  A  D  N

361  TCGACAATAT GTACCATGCG TCGATTCTGG AGCTGGAAGA AAGTCGCCTC GCAAGGTACT
      R  Q  Y  V  P  C  V  D  S  G  A  G  R  K  S  P  R  K  V  L

421  TCTTAGATCT ATTGAAGCTG TGTTTAACGG AAGCTTCAGC GGAAATAATA GGAATGTTCG
      L  R  S  I  E  A  V  F  N  G  S  F  S  G  N  N  R  N  V  R

481  TGGATTTCTC TACGTATCGA TCAGAGACGA TGACGGAGAA ATGCGTCCAG TACTCATAGT
      G  F  L  Y  V  S  I  R  D  D  D  G  E  M  R  P  V  L  I  V

541  ACCATTCGGA GGATATGGAT ATCATAATGA TTTTTATTAT TTCGAAGGGA AGGGGAAAGT
      P  F  G  G  Y  G  Y  H  N  D  F  Y  Y  F  E  G  K  G  K  V

601  TGAATGTGAT ATATCATCAG ATTATGTTGC GCCAGGAATA GATTGGAGCA GAGACATGGA
      E  C  D  I  S  S  D  Y  V  A  P  G  I  D  W  S  R  D  M  E

661  AGTTAGTATT AGTAACAGCA ACAACTGTAA TGAATTATGT GATCTGAAGT GTTATGTTGT
      V  S  I  S  N  S  N  N  C  N  E  L  C  D  L  K  C  Y  V  V

721  TTGTTCGTTA AGAATCAAGG AATAAAAGTT GTGCTGTAAT GTTAATTAAT AAAACGTATA.
      C  S  L  R  I  K  E  *

781  TTTGGGAAAT TGATAGTTGT ATAAAACATA CAACACACTA TGAAATACAA GACGCTATGA

841  CAAATGTACG GGTATCTGAA TGAGTTTTAG TATCGCTTAA GGGCCGCAGG CCCGTTAAAA

901  ATAATAATCG AATTATAAAC GTTAGATAAT AATCAGAGAT AGGTGATCAG ATAATATAAA

961  CATAAACGAA GTATATGCCG GTACAATAAT AAAATAAGTA ATAACAAAAA AAATATGTAT

1021 ACTAATCTCT GATTGGTTCA GGAGAAAGGC CCACCAACTA AAAGGTGGGG AGAATGTCCC

1081 GATGACGTA
```

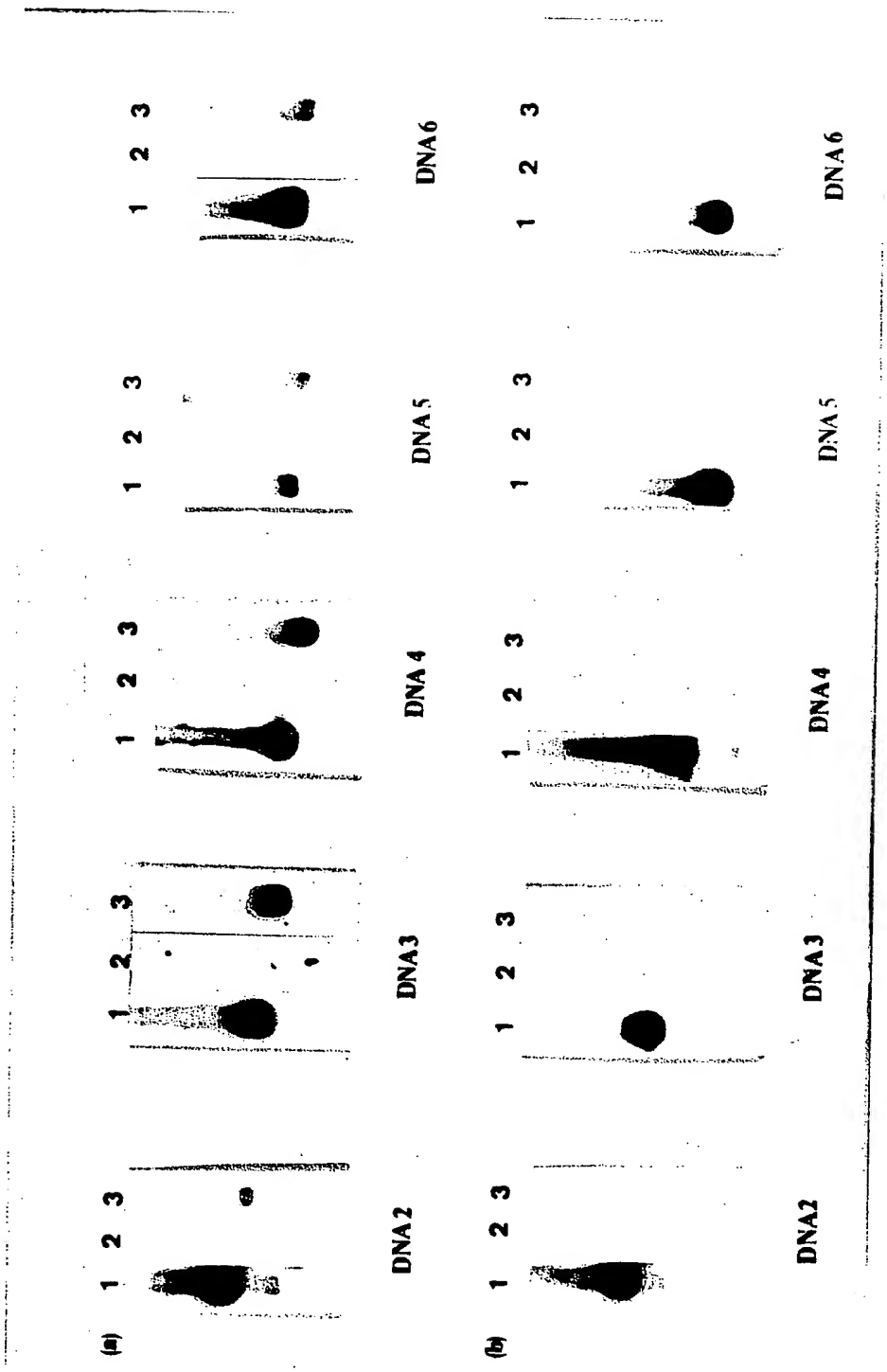


FIG. 2

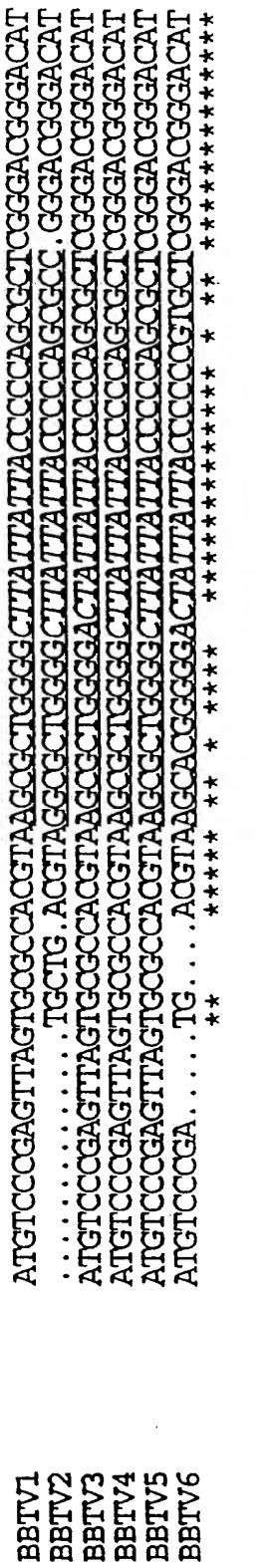


FIG. 3

```
BBTV1  .....CACACTATGACAAAAGTAYGGGTATCTGATTGGGTTATCTTAACGATCT.AGGGCCGTAGGCCCGT
BBTV2  GAAATACAACACGCTATGAAATACAAGACGCTATGACAAATGTAYGGGWATMTGATTGTGTA.TCTTAACG.TATAAGGGCCGCAGCCCGT
BBTV3  AACATACAACACGCTATGAAATACAAGACGCTATGACAAAAGTACTGGTATATGATTAGGTA.TCCTAACGATCTA.GGGCCGAAGCCCCGT
BBTV4  AACATACAACACGCTATGAAATACAAGACGCTATGACAAAAGTACTGGTATATGATTAGGTA.TCCTAACGATCTA.GGGCCGAAGCCCCGT
BBTV5  AACATACAACACACTATAAAATACAACACACTATAACAAATGTACGGGTATTTGATTTGGCTATATTAACCCCTTAAGGGCCGAAGCCCCGT
BBTV6  AACATACAACACACTATGAAATACAAGACGCTATGACAAATGTACGGGTATCTGAATGAGTTTTTAGTA.TCGCTTAAGGGCCGCAGCCCCGT
       ▲▲▲▲▲▲▲▲▲▲ ▲▲▲▲ ▲▲▲▲▲▲▲▲ ** **** ***** **** ***** *****  *    *  **      ** ***** ********
```

FIG. 4

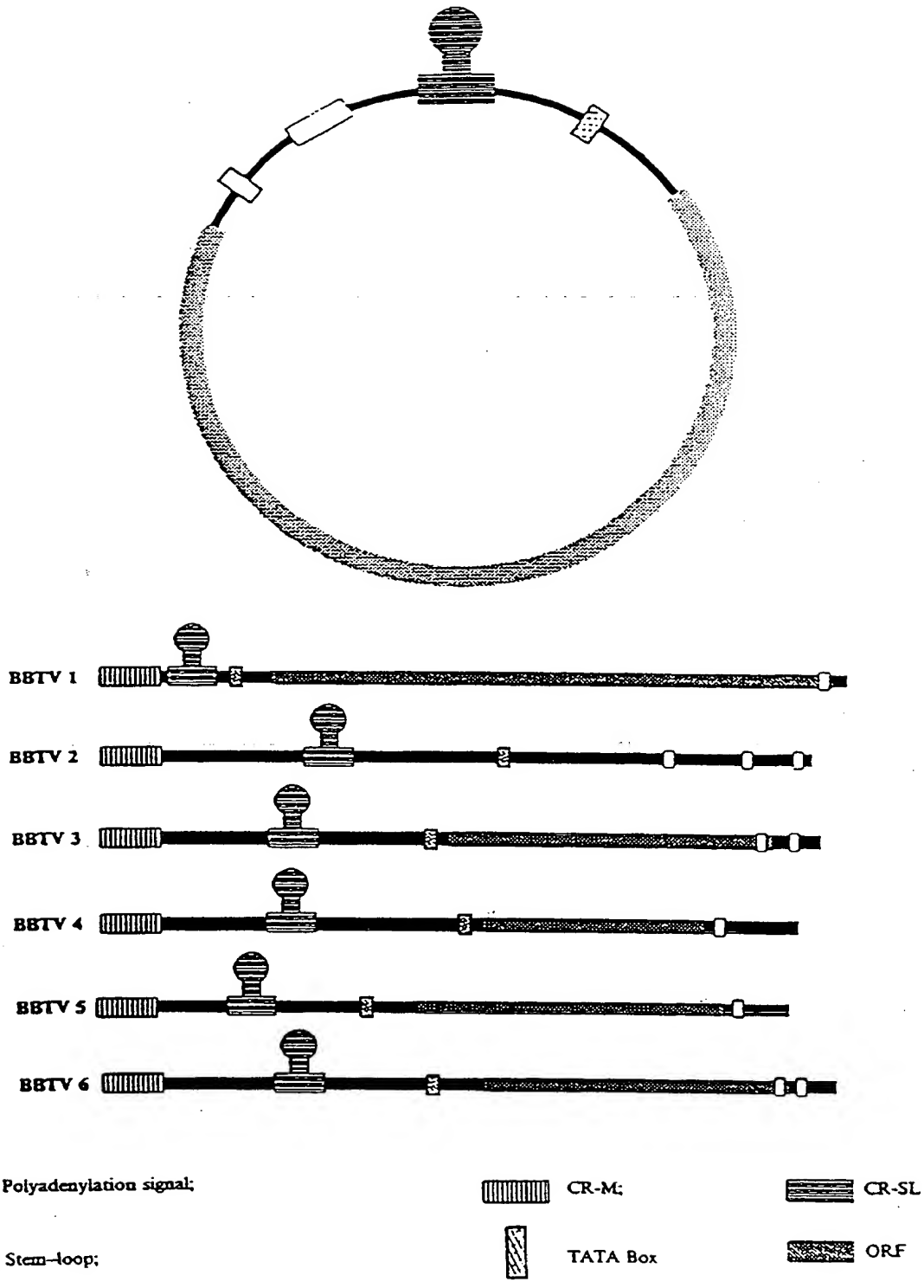

BBTV 1
BBTV 2
BBTV 3
BBTV 4
BBTV 5
BBTV 6
Polyadenylation signal;
Stem-loop;
CR-M;
TATA Box
CR-SL
ORF

FIG. 5

FIG. 6

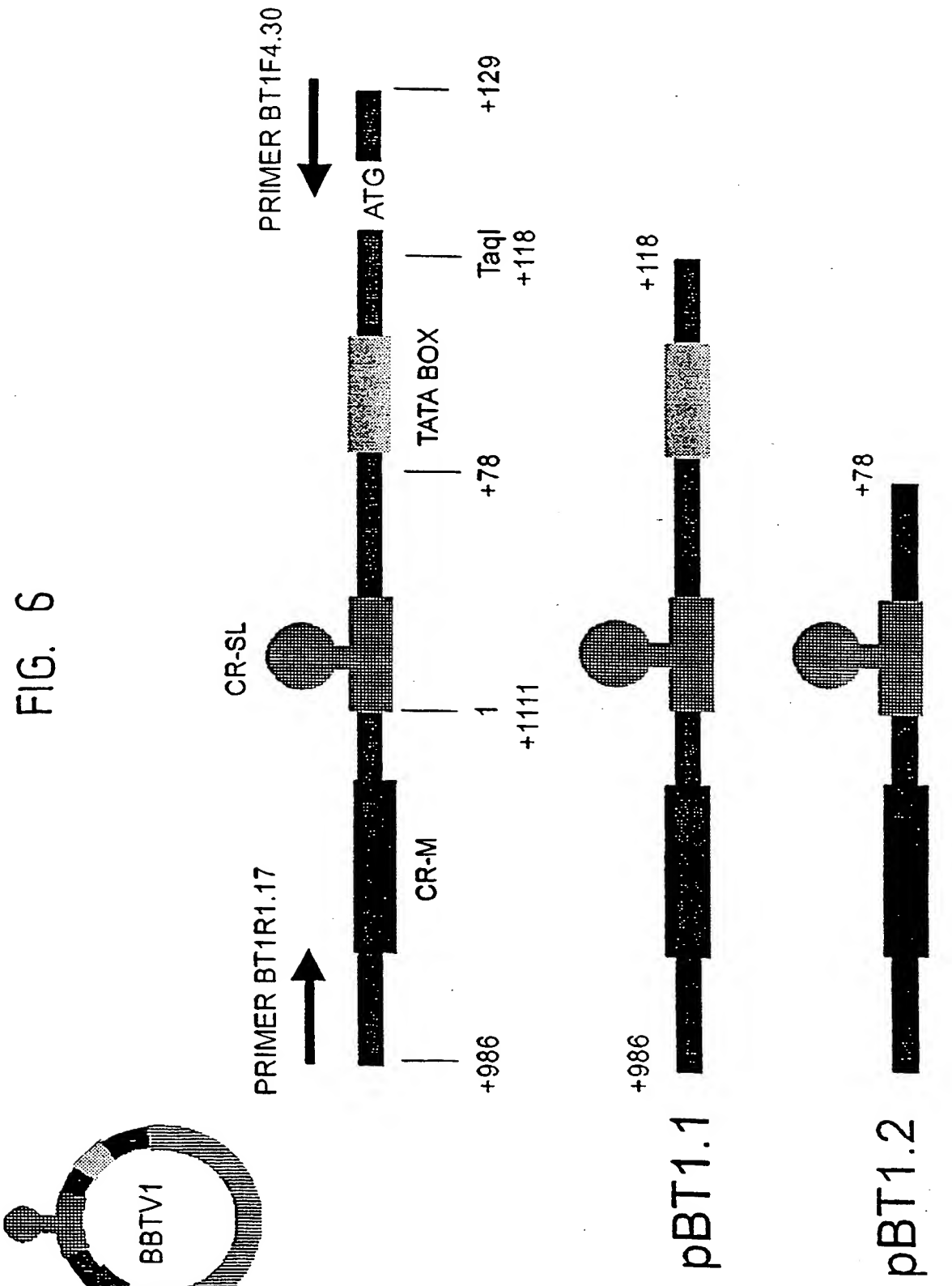


FIG. 7

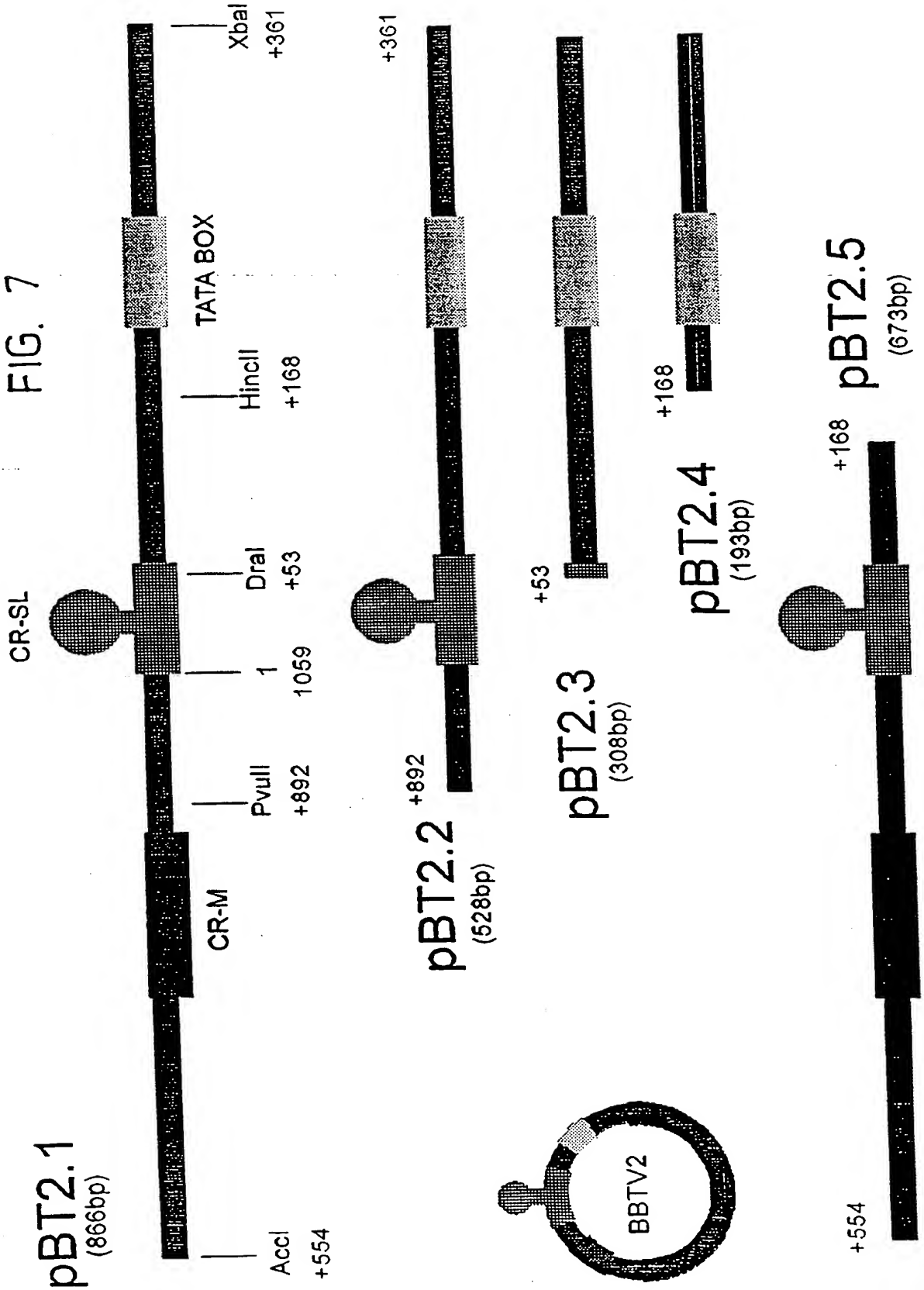
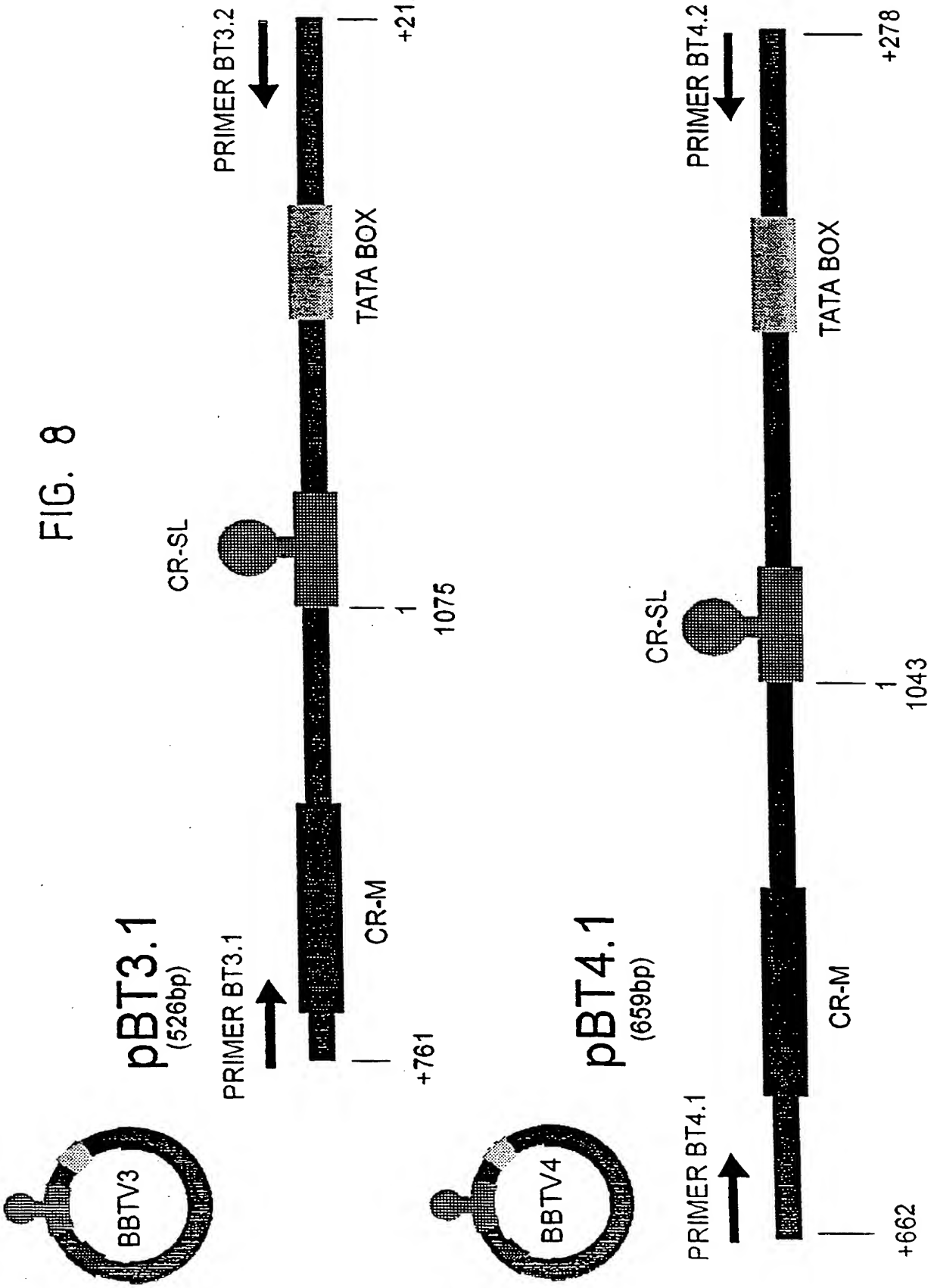


FIG. 8



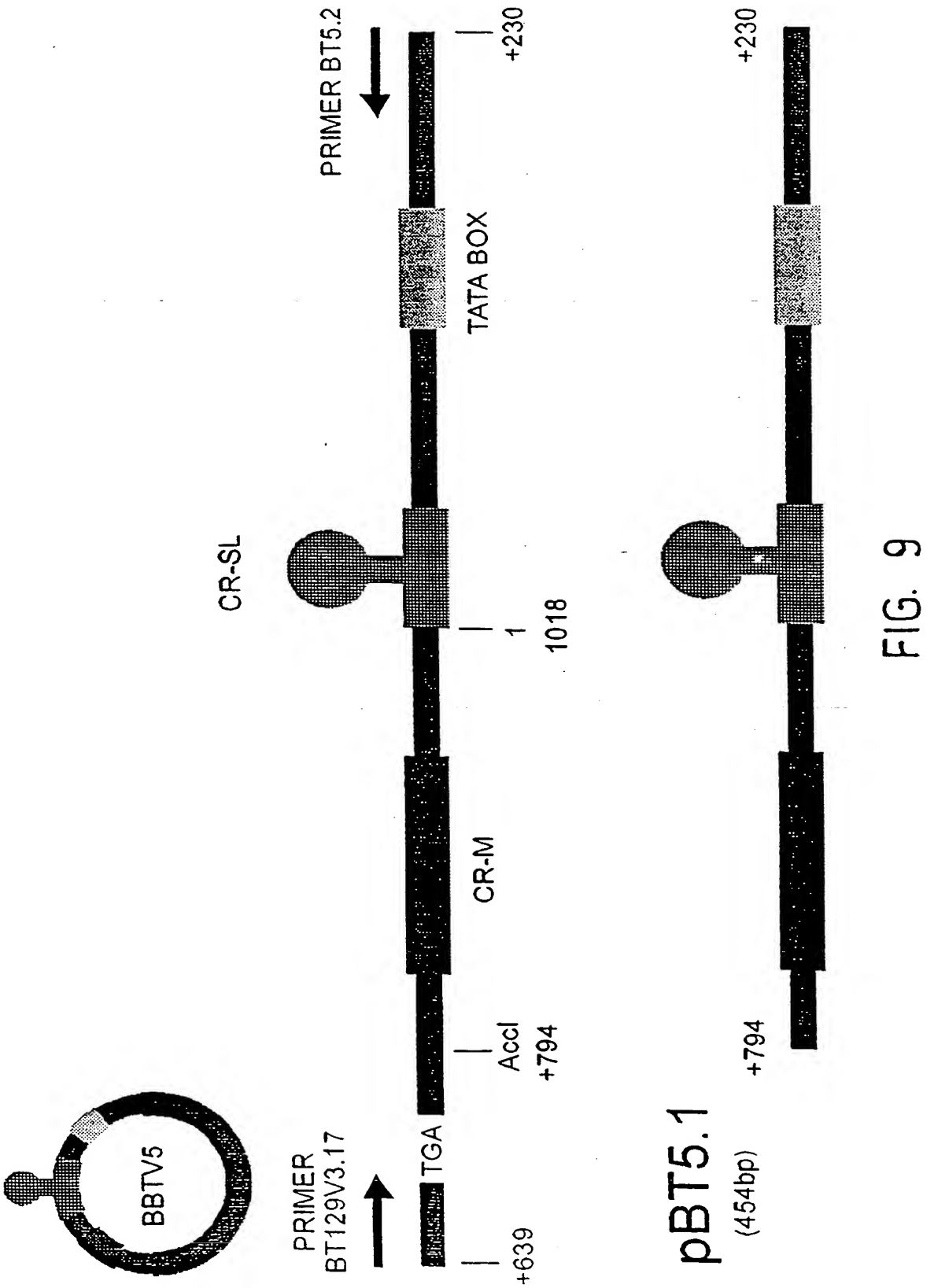


FIG. 9

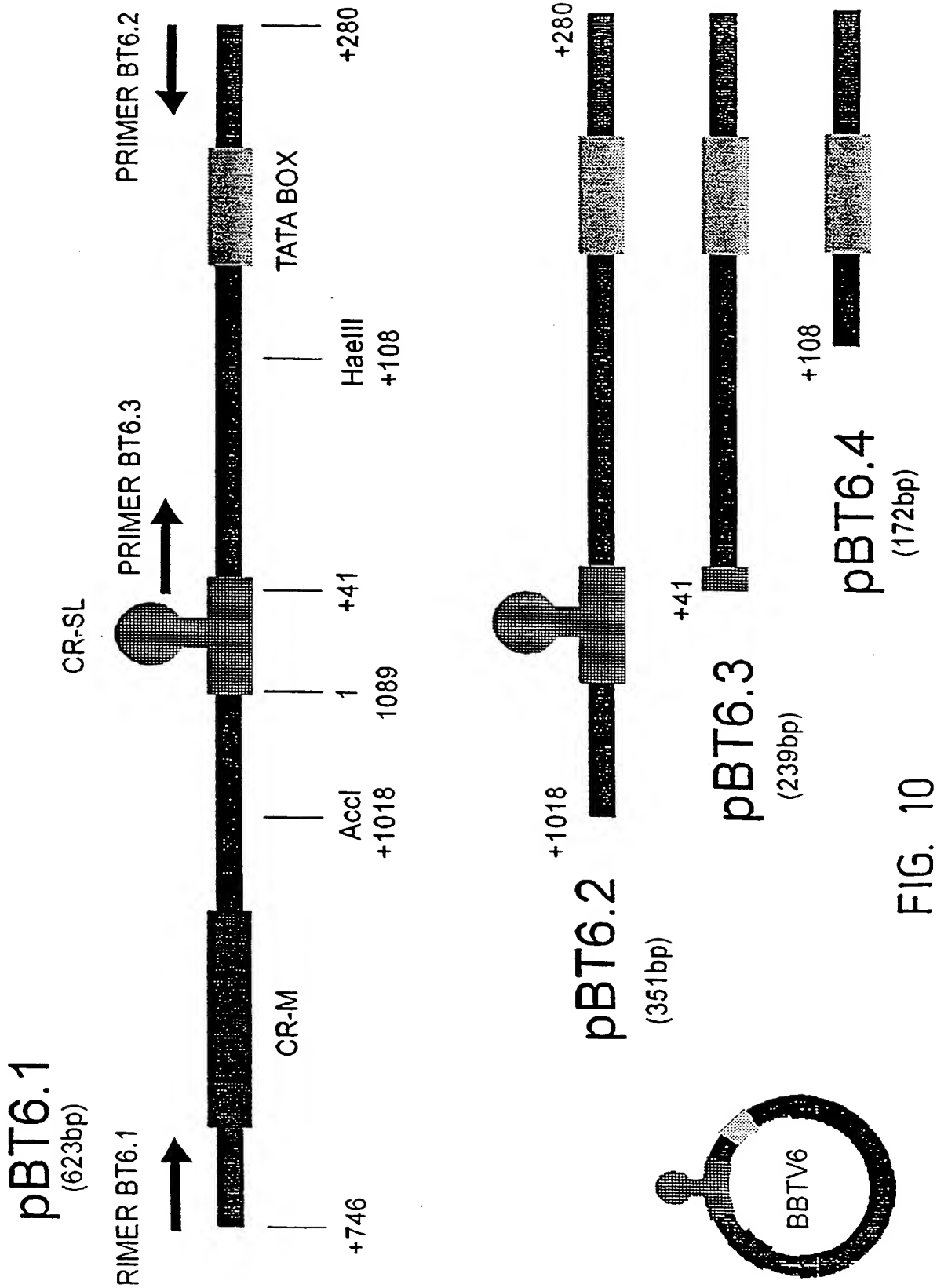


FIG. 10

```
            1                                                   50
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    .......... .......... .......... .......... ..........
bbtvpro3    .......... .......... .......... .......... ..........
bbtvpro4    .......... .......... .......... .......... ..........
bbtvpro6    .......... .......... .......... .......... ..........
bbtvpro2    TATACGGAAC GTATACTAAC GTAAAAATTA AATGATAGGC GAAGCATGAT

            51                                                  100
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    .......... .......... .......... .......... ..........
bbtvpro3    .......... .......... .......... .......... ..........
bbtvpro4    ...TACACGG TATATTAATA TACGAAATAT AAATGGGTAT TGATGTAAAT
bbtvpro6    .......... .......... .......... .......... ..........
bbtvpro2    TAACAGGTGT TTAGGTATAA TTAACATAAT TATGAGAAGT AATAATAATA

            101                                                 150
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    .......... .......... .......... .......... ..........
bbtvpro3    .......... .......... .........T ATGTTTATGT AAACATAAAC
bbtvpro4    GATCATACAT AATATATGTA TGATAATGAA ACATATTGTA ATATGTGAAT
bbtvpro6    .......... .....AAGTT GTGCTGTAAT GTTAATTAAT AAAACGTATA
bbtvpro2    CGGAAAATGA ATAAGTATGA GGTGAAAGAG GAGATATTAG AATATTTAAA

            151                                                 200
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    .......... .......... ...TGTAATA TCCATTATCA TCAATAAAAT
bbtvpro3    TATTGTATGG AATGAAATCC AAATAACATA CAACACGCTA TGAAATACAA
bbtvpro4    TGTAAACGAG AGTTGTATGT ATAAAACATA CAACACGCTA TGAAATACAA
bbtvpro6    TTTGGGAAAT TGATAGTTGT ATAAAACATA CAACACACTA TGAAATACAA
bbtvpro2    AACCCAATTA TATTATTTTG GAACGAAATA CAACACGCTA TGAAATACAA
```

FIG. 11

```
            201                                                        250
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    AATGGAATGT TGATTATGTA TTTATCATAA ATACATAATG GTATACGTAT
bbtvpro3    GACGCTATGA CAAAAGTACT GGTATATG.A TTAGGTATCC TAACGATCTA
bbtvpro4    GACGCTATGA CAAAAGTACT GGTATATG.A TTAGGTATCC TAACGATCTA
bbtvpro6    GACGCTATGA CAAATGTACG GGTATCTGAA TGAGTTTTAG TATCGCTTAA
bbtvpro2    GACGCTATGA CAAATGTACG GGAATATG.A TTGTGTATCT TAACGTATAA

            251                                                        300
bbtvpro1    .........A CAAGTAATGA CTTTACAGCG CACGCTCC.. ..........
bbtvpro5    AGCATAAAAT ACATTAACCA ACATACAACA CACTATAA.. ..........
bbtvpro3    GGGCCGAAGG CCCGTGAGCA ATATGCGTCG AAATA.AT.. ..........
bbtvpro4    GGGCCGAAGG CCCGTGAGCA ATATGCGTCG AAATA.AT.. ..........
bbtvpro6    GGGCCGCAGG CCCGTTAAAA ATAATAATCG AATTATAA.. ..........
bbtvpro2    GGGCCGCAGG CCCGTCAAGT TGAATGAACG GTCCAGATTA ATTCCTTAGC

            301                                                        350
bbtvpro1    GACAAAAGCA CACTATGACA AAAGTACGGG TATCTGATTG GGTTATCTTA
bbtvpro5    AATACAA.CA CACTATAACA AATGTACGGG TATTTGATTG GGCTATATTA
bbtvpro3    GTTTAACAAA CAAATATACA TGATACGGAT AGTTGAATAC ATAAACAACG
bbtvpro4    GTTTAACAAA CAAATATACA TGATACGGAT AGTTGAATAC ATAAACAACG
bbtvpro6    ACGTTAGATA ATAATCAGAG ATAGGTGATC AGATAATATA AACATAAACG
bbtvpro2    GACGAAGAAA GGAATCTTAA AGGGGACCAC ATTAAAGACA GCTGTCATTG

            351                                                        400
bbtvpro1    A.CGATCTAG GGCCGTAGGC CCG..... .......... ..........
bbtvpro5    ACCCCTTAAG GGCCGAAGGC CCGTTTAAAT ATGTGTTGGA CGAAGTCCAA
bbtvpro3    AGGTATACAA TACAACAAAC TGTTGTAAAG AAATA..AAA AATAAGAAGA
bbtvpro4    AGGTATACAA TACAACAAAC TGTTGTAAAG AAATA..AAA AATAAGAAGA
bbtvpro6    AAGTATATGC CGGTACAATA ATAAAATAAG TAATAACAAA AAAAATATGT
bbtvpro2    ATTAAATAAA TAATATAATA ACCAAAAGAC CTTTGTACCC TTCCTAATGA

            401                                                        450
bbtvpro1    .......... .......... .......... ..TGAGCAAT GAAC......
bbtvpro5    ACACAAAAAA GTAAGCAGAA CAA.CGGAAT AATATGAGCT GGCA......
bbtvpro3    GAGAGTATAT TTGTGTCGGA TAAGCATCAC ACCCACCACT TTAG......
bbtvpro4    GATAGTATAT TTGTGTTGGA TAAGCCTTGC AACCACCACT TTAG......
bbtvpro6    ATACTAATCT CTGATTGGTT CAGGAGAAAG GCCCACCAAC TAAA......
bbtvpro2    TGACGTATAG GGGTGTCCCG ATGTAATTTA ACATAGCTCT GAAAAGAGAT

            451                                                        500
bbtvpro1    .........G GCGAGATCAG ATGTCCCGAG TTAGTGCG.. ..........
bbtvpro5    .........A CGTAGGGTCC ATGTCCCGAG TTAGTGCG.. ..........
bbtvpro3    .........T GGTGGGCCAG ATGTCCCGAG TTAGTGCG.. ..........
bbtvpro4    .........T GGTGGGCCAG ATGTCCCGAG TTAGTGCG.. ..........
bbtvpro6    .........A GGTGGGGAGA ATGTCCCGA. .......... ..........
bbtvpro2    ATGGGCCGTT GGATGCCTCC ATCGGACGAT GGAGGTTGAA TGAACTTCTG
```

```
                501                                                     550
bbtvpro1  .CCACGTAAG CGCTGGGGCT TATTATTACC CCCAGCGCTC GGGACGGGAC
bbtvpro5  .CCACGTAAG CGCTGGGGCT TATTATTACC CCCAGCGCTC GGGACGGGAC
bbtvpro3  .CCACGTAAG CGCTGGGGAC TATTATTACC CCCAGCGCTC GGGACGGGAC
bbtvpro4  .CCACGTAAG CGCTGGGGCT TATTATTACC CCCAGCGCTC GGGACGGGAC
bbtvpro6  .TGACGTAAG CACGGGGGAC TATTATTACC CCCCGTGCTC GGGACGGGAC
bbtvpro2  CTGACGTAGG CGCTGGGGCT TATTATTACC CCCAGCGC.C GGGACGGGAC

                551                                                     600
bbtvpro1  ATTTGCATCT ATAAATAGAC .......... .CTCCCCCCT CTCCATTACA
bbtvpro5  ATCACGTGCA ACTAACAGAC GCACGTGAGA ATGCAGTAGC TTGCAGCGAA
bbtvpro3  ATGGGCTAAT GGA....... ....TTGTGG ATATAGGGCC CAAAGGGCCC
bbtvpro4  ATCACGTGCG TCAACAAATG CACGTGACTG ATATAAGGGA CATAACGGGT
bbtvpro6  ATTGACGTCAG CAAGGATTAT AATGGGCTTT TTATTAGCCC ATTTATTGAA
bbtvpro2  ATGGGCTTTT TAAATGGGCT TTGCGAGTTT GAACAGTTCA GTATCTTCGT

                601                                                     650
bbtvpro1  AGATCATCAT CGACGACAGA .......... .......... ..........
bbtvpro5  AGATAGACGT CAACATCAAT AAAGAAGAAG GAATATTCTT TGCTTCGGCA
bbtvpro3  GTTTAGATGG GTTTTGGGCT CATGGGCTTT ATCCAGAAGA CCAAAAACAG
bbtvpro4  TTAGATAACG GTTTATGCGG ATTAGAATAT AACGTCACGT GTGAAAGCCG
bbtvpro6  TT..GGGCCG GGTTTTGTCA TTTTACAAAA GCCCGGTCCA GGATAAGTAT
bbtvpro2  TATTGGGCCA ACCCGGCCCA ATAATTAAGA GAACGTGTTC AAATTCGTGG

                651                                                     700
bbtvpro1  .......... .......... .......... .......... ..........
bbtvpro5  CGAAGCAAAG GGTATAGATA TTTGTTCGAG ATGCGAAAAT GGAGGCTATT
bbtvpro3  GCGGGAACCG TCCCAA.... .......... ......ATTC AAACTTCGAT
bbtvpro4  AAAGGCACGT GACGAAGACA AATGGATTGA ATAAACATTT GACGTCCGGT
bbtvpro6  AATGTCACGT GCCGAATTAA AAGGTTGCTT CGCCACGAAG AAACCTAATT
bbtvpro2  TATGACCGAA GGTCAAGGTA ACCGGTCAAC ATTATTCTGG CTTGCGCAGC

                701                                                     750
bbtvpro1  .......... .......... .......... .......... ..........
bbtvpro5  TAAACCTGAT GGTTTTGTGA TTTCCGAAAT CACTCGTCGG AAGAGAA...
bbtvpro3  ..TGCTTGCC CTGCAACGCA TCTAGAAGTC TATAAATACC AGTGTCTAGA
bbtvpro4  ..AGCTTCCG AAGGAAGTAA GCTTCGCGGC GAAGCAAACC ATTTATATAT
bbtvpro6  TGAGGTTGCG TATTCAATAC GCTACCGAAT ATCTATTAAT ATGTGAGTCT
bbtvpro2  AAGATACACG AATTAATTTA TTAATTCGTA GGACACGTGG ACGGACCGAA

                751                                                     800
bbtvpro1  .......... .......... .......... .......... ..........
bbtvpro5  .......... .......... .......... .......... ..........
bbtvpro3  TAG....... .......... .......... .......... ..........
bbtvpro4  TTGCGTAGGC TTGCGGCCTA TAAATAGGAC GCAGCTAA.. ..........
bbtvpro6  CTGCCGAAAA AAATCAGAGC GAAAGCGGAA GGCAGAAGCG ..........
bbtvpro2  ATACTCTTGC ATCTCTATAA ATACCCTAAT CCTGTCAAGG ATAATTGCTC
```

```
            801                                                       850
bbtvpro1    .......... .......... .......... .......... ..........
bbtvpro5    .......... .......... .......... .......... ..........
bbtvpro3    .......... .......... .......... .......... ..........
bbtvpro4    .......... .......... .......... .......... ..........
bbtvpro6    .......... .......... .......... .......... ..........
bbtvpro2    TCTCTCTTCT GTCAAGGTGG TTGTGCTGAG GCGGAAGATC GCCAGCGGCG

            851                  871
bbtvpro1    .......... .......... .
bbtvpro5    .......... .......... .
bbtvpro3    .......... .......... .
bbtvpro4    .......... .......... .
bbtvpro6    .......... .......... .
bbtvpro2    ATCGTCGGAA CGACCTGCAT A
```

ATAAAACGAAGGCGATGAATAGCTGGAGAACTTCTTTCAGTGCTTGGACATCAGAGGTGG
AGAATATCATGGCGCAGCCATGTCATCGGAGAATAATTTGGGTCTATGGCCCAAATGGAG
GAGAAGGAAAGACAACGTATGCAAAACATCTAATGAAGACGAGAAATGCGTTTTATTCTC
CAGGAGGAAAATCATTGGATATATGTAGACTGTATAATTACGAGGATATTGTTATATTTG
ATATTCCAAGATGCAAAGAGGATTATTTAAATTATGGGTTATTAGAGGAATTTAAGAATG
GAATAATTCAAAGCGGGAAATATGAACCCGTTTTGAAGATAGTAGAATATGTCGAAGTCA
TTGTAATGGCTAACTTCCTTCCGAAGGAAGGAATCTTTTCTGAAGATCGAATAAAGTTGG
TTTCTTGCTGAACAAGTAATGACTTTACAGCGCACGCTCCGACAAAAGCACACTATGACA
AAAGTACGGGTATCTGATTGGGTTATCTTAACGATCTAGGGCCGTAGGCCCGTGAGCAAT
GAACGGCGAGATCAGATGTCCCGAGTTAGTGCGCCACGTAAGCGCTGGGGCTTATTATTA
CCCCCAGCGCTCGGGACGGGACATTTGCATCTATAAATAGACCTCCCCCCTCTCCATTAC
AAGATCATCATCGACGACAGAATGGCGCGATATGTGGTATGCTGGATGTTCACCATCAAC
AATCCCACAACACTACCAGTGATGAGGGATGAGATAAAATATATGGTATATCAAGTGGAG
AGGGGACAGGAGGGTACTCGTCATGTGCAAGGTTATGTCGAGATGAAGAGACGAAGCTCT
CTGAAGCAGATGAGAGGCTTCTTCCCAGGCGCACACCTTGAGAAACGAAAGGGAAGCCAA
GAAGAAGCGCGGTCATACTGTATGAAGGAAGATACAAGAATCGAAGGTCCCTTCGAGTTT
GGTTCATTTAAATTGTCATGTA

FIG. 12

(a)

AGTTGTGCTGTAATGTTAATTAATAAAACGTATATTTGGGAAATTGATAGTTGTATAAAACATACAACACACTAT
GAAATACAAGACGCTATGACAAATGTACGGGTATCTGAATGAGTTTTAGTATCGCTTAAGGGCCGCAGGCCCGTT
AAAAATAATAATCGAATTATAAACGTTAGATAATAATCAGAGATAGGTGATCAGATAATATAAACATAAACGAAG
TATATGCCGGTACAATAATAAAATAAGTAATAACAAAAAAAATATGTATACTAATCTCTGATTGGTTCAGGAGAA
AGGCCCACCAACTAAAAGGTGGGGAGAATGTCCCGATGACGTAAGCACGGGGGACTATTATTACCCCCCGTGCTC
GGGACGGGACATGACGTCAGCAAGGATTATAATGGGCTTTTTATTAGCCCATTTATTGAATTGGGCCGGGTTTTG
TCATTTTACAAAAGCCCGGTCCAGGATAAGTATAATGTCACGTGCCGAATTAAAAGGTTGCTTCGCCACGAAGAA
ACCTAATTTGAGGTTGCGTATTCAATACGCTACCGAATATCTATTAATATGTGAGTCT
CTGCCGAAAAAAATCAGAGCGAAAGCGGAAGGCAGAAGC

(b)

GTATACTAATCTCTGATTGGTTCAGGAGAAAGGCCCACCAACTAAAAGGTGGGGAGAATGTCCCGATGACGTA AG
CACGGGGGACTATTATTACCCCCCGTGCTCGGGACGGGACATGACGTCAGCAAGGATTATAATGGGCTTTTTATT
AGCCCATTTATTGAATTGGGCCGGGTTTTGTCATTTTACAAAAGCCCGGTCCAGGATAAGTATAATGTCACGTGC
CGAATTAAAAGGTTGCTTCGCCACGAAGAAACCTAATTTGAGGTTGCGTATTCAATACGCTACCGAATATCTATT
AATATGTGAGTCTCTGCCGAAAAAAATCAGAGCGAAAGCGGAAGGCAGAAGC

(c)

CATGACGTCAGCAAGGATTATAATGGGCTTTTTATTAGCCCATTTATTGAATTGGG CCGGGTTTTGTCATTTTAC
AAAAGCCCGGTCCAGGATAAGTATAATGTCACGTGCCGAATTAAAAGGTTGCTTCGCCACGAAGAAACCTAATTT
GAGGTTGCGTATTCAATACGCTACCGAATATCTATTAATATGTGAGTCTCTGCCGAAAAAAATCAGAGCGAAAGC
GGAAGGCAGAAGC

(d)

CCGGGTTTTGTCATTTTACAAAAGCCCGGTCCAGGATAAGTATAATGTCACGTGCCGAATTAAAAGGTTGCTTCG
CCACGAAGAAACCTAATTTGAGGTTGCGTATTCAATACGCTACCGAATATCTATTAATATGTGAGTCTCTGCCGA
AAAAAATCAGAGCGAAAGCGGAAGGCAGAAGC

FIG. 13

FIG. 14

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00335

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/11 15/82 5/10 A01H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC6 : C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, CA
Search Terms: Banana( ) Bunchy( ) Top( ) virus or BBTV

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of General Virology (1993) vol. 74 Harding et al., "Nucleotide sequence of one component of the banana bunchy top virus genome contains a putative replicase gene" pages 323-328 see abstract	
A	Journal of General Virology (1994) vol. 75 Karan et al., "Evidence for two groups of banana bunchy top virus isolates". P 3541-3546, see abstract, p 3544 left column	
A	Archives of Virology (1994) vol. 137 Burns et al., "Evidence that banana bunchy top virus has a multiple component genome". pages 371-380 see results p 373 & 374	

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 July 1996	15 AUG 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer J.H. CHAN Telephone No.: (06) 283

Form PCT/ISA/210 (second sheet) (July 1992) copvak

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00335

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of General Virology (1995) vol. 76 Burns et al., "The genome organization of banana bunchy top virus: analysis of six ssDNA components". pages 1471-1482 see entire document Especially abstract	